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MAINTENANCE OF VISUAL SENSITIVITY IN THE *DROSOPHILA* EYE

A Dissertation Presented

by

LINA NI

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

January 15, 2010

NEUROSCIENCE

**MAINTENANCE OF VISUAL SENSITIVITY
IN THE *DROSOPHILA* EYE**

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LINA NI

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January 15, 2010

Dedicated to my beloved family

My parents

Ziliang Ni

GuiHua Meng

My husband

Jianhong Ou

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ABSTRACT

High visual sensitivity is a common but important characteristic of animal eyes. It is especially critical for night vision. In animal eyes, photoreceptors are the first to receive the incoming rays of light and they convert the light signals to electrical signals before passing the information to interneurons in the eye and finally to the brain.

To function in dim light conditions, photoreceptors have developed high sensitivities to light. It is reported that both mammalian rod photoreceptors and *Drosophila* photoreceptors can detect single photons.

The high sensitivities of photoreceptors largely depend on a high content of rhodopsin, a light-stimulated G protein-coupled receptor (GPCR), in light sensory organelles, outer segments in mammals and rhabdomeres in *Drosophila*. Two shared characteristics, the tightly packed photoreceptive membrane and the high concentration of rhodopsin in the membrane, work together to enable the photoreceptors to achieve the high content of rhodopsin in photosensory organelles in both mammals and *Drosophila*. In this thesis, I have used the *Drosophila* eye as a model system to study the molecular mechanisms required for the maintenance of these two characteristics.

In the second chapter, I present a new molecular mechanism of preventing G_q-mediated rhabdomeral degeneration. A new gene named *tadr* (for *t*orn *a*nd *d*iminished *r*habdomeres), when mutated, leads to visual sensitivity reduction and photoreceptor degeneration. Degeneration in the *tadr* mutant is characterized by shrunken and disrupted rhabdomeres. The TADR protein interacts *in vitro* with the major light receptor Rh1 rhodopsin, and genetic reduction of the Rh1 level suppresses the *tadr*-induced degeneration, suggesting the degeneration is Rh1-dependent. Nonetheless, removal of phospholipase C (PLC), a key enzyme in phototransduction, and that of Arr2 fail to inhibit rhabdomeral degeneration in

the *tadr* mutant background. Biochemical analyses reveal that, in the *tadr* mutant, the G_q protein of Rh1 is defective in dissociation from the membrane during light stimulation. Importantly, reduction of G_q level by introducing a hypomorphic allele of $G_{\alpha q}$ gene greatly inhibits the *tadr* degeneration phenotype. These results may suggest that loss of a potential TADR-Rh1 interaction leads to an abnormality in the G_q signaling, which in turn triggers rhabdomeral degeneration independent of the PLC phototransduction cascade. We propose that TADR-like proteins may also protect photoreceptors from degeneration in mammals including humans.

In the third chapter, I present a *Drosophila* CUB- and LDLa-domain transmembrane protein CULD that counteracts the visual arrestin Arr1-mediated endocytosis to retain rhodopsin in rhabdomeral membrane. CULD is mostly localized in rhabdomeres, but is also detected in scarce rhodopsin endocytic vesicles that contain Arr1. An intracellular region of CULD interacts with Arr1 *in vitro*. In both *culd* mutant and knockdown flies, a large amount of rhodopsin is mislocalized in the cell body of photoreceptors through light-dependent, Arr1-mediated endocytosis, leading to reduction of photoreceptor sensitivity. Expressing a wild-type CULD protein in photoreceptors, but not a mutant variant lacking the Arr1-interacting site, rescues both the rhodopsin mislocalization and the low sensitivity phenotypes. Once rhodopsin has been internalized in adult mutant flies, it is reversed only by expression of CULD but not by blocking endocytosis, suggesting that CULD promotes recycling of endocytosed rhodopsin to the rhabdomere. Our results demonstrate an important role of CULD in the maintenance of membrane rhodopsin density and photoreceptor sensitivity. We propose that a common cellular function of CUB- and LDLa-domain proteins, in both mammals and invertebrates, is to concentrate receptors including GPCRs in particular regions of cell membrane.

In summary, the work addressed in this thesis has identified new molecular mecha-

nisms underlying the maintenance of visual sensitivity in *Drosophila*, either through preventing G_q-mediated rhabdomeral degeneration or through antagonizing arrestin-mediated rhodopsin endocytosis. This work has advanced our understanding of visual biology and the general regulatory mechanisms of GPCR signaling, and may provide valuable clues to pathologic studies of human retinal degeneration disorders.

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LIST OF ABBREVIATION

5'-GMP	guanosine 5'-monophosphate
AChR	acetylcholine receptor
AD	Alzheimer's disease
ADRP	autosomal dominant retinitis pigmentosa
AngII	angiotensin II
ARF	ADP-ribosylation factor
AT ₂	angiotensin type 2 receptor
cGMP	cyclic guanosine monophosphate
CUB	complement C1r/C1s, uegf, bone morphogenic protein 1
CULD	CUB and LDLadomain transmembrane protein
DAG	diacylglycerol
EM	electron microscopy
EMS	ethyl methanesulfonate
ERG	electroretinogram
G _t	transducin
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
IP ₃	inositol-1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
LDLa	low-density lipoprotein receptor domain class A
MAPK	mitogen-activated protein kinase
MBP	maltose binding protein
MOR	mu opioid receptor

NETO	neuropilin tolloid-like
NMDAR	N-methyl-D-aspartate receptor
PDA	prolonged depolarization afterpotential
PDE	phosphodiesterase
PIP ₂	phosphatidylinositol-4,5-biphosphate
PLC	phospholipase C
PUFA	polyunsaturated fatty acid
RNAi	RNA interference
ROS	rod outer segments
RP	retinitis pigmentosa
SEM	standard error of the mean
SLC7	solute carrier 7
TADR	torn and diminished rhabdomeres
TB	toluidine blue staining
TRP	transient receptor potential
TRPL	transient receptor potential-like
WT	wild type

CHAPTER I: INTRODUCTION

1.1 Visual sensitivity

We use sensory systems to get to know the world. Among them, vision is of the most importance. It allows animals to navigate in the world; to judge the speed and distance of objects; and to identify food, other species and familiar or unfamiliar members of the same species (Squire et al., 2003).

Vision begins with light entering the eye. In mammals, light is projected onto the retina, a three-layered thin neural structure with five types of neurons. Among them, photoreceptors are the first to receive the incoming rays of light. Photoreceptors convert the light signals into transmittable electrical signals before passing the information to interneurons in the eye and finally to the brain (Squire et al., 2003).

To work in dim light, animal photoreceptors have developed utmost sensitivities to light. Mammalian eyes have two types of photoreceptors: rods and cones. While cones function during daytime and are responsible for color vision, rods are specialized for nighttime vision (Squire et al., 2003). Loss of rod cells causes night blindness in several human retinal disorders, such as retinitis pigmentosa (RP) that affects about 400,000 Americans (www.preventblindness.org).

It is reported that rod cells are capable of detecting single photons (Rieke and Baylor, 1996). Such a high sensitivity largely depends on a high amount of rhodopsin molecules in the outer segment, the light sensory organelle of the rod photoreceptor. The following two characteristics enable the rod outer segment to contain the high amount of rhodopsin:

- 1) The tightly packed membrane structure of the outer segment. It consists of a plasma membrane that encloses a stack of about 1,000 closely spaced optic discs (Fig.1.1B). The membrane structure dramatically increases the sensory membrane area so that a huge amount of rhodopsin molecules can be packed in (Hardie and Raghu, 2001; Squire et al.,

2003). Degeneration of the outer segment leads to reduction of rod sensitivity (Lem and Fain, 2004). 2) The high rhodopsin concentration in the optic disc membrane. It has been reported that rhodopsin molecules occupy about 50% of the membrane (Palczewski, 2006). Decrease of rhodopsin concentration in the membrane reduces rod sensitivity.

In addition to relying on the high rhodopsin amount, the utmost sensitivities of photoreceptors also depend on the exceedingly low spontaneous activity of rhodopsin and downstream signaling molecules, which sets the limit on the absolute sensitivity of phototransduction (Rao et al., 1994; Dizhoor et al., 2008; Tsang et al., 2007).

In this thesis, we are studying the mechanisms required to maintain the high amount of rhodopsin in photoreceptor sensory organelles by discussing the following two questions.

How do photoreceptors maintain the membrane structure of light sensory organelles?

How do photoreceptors maintain the concentration of rhodopsin in photoreceptive membrane?

We will use *Drosophila* as a genetic system to study these questions.

1.2 *Drosophila* visual system

The *Drosophila* compound eye is made up of about 800 units, known as ommatidia (Fig. 1.1C). Each ommatidium contains 8 photoreceptors. Six peripheral photoreceptors, R1-R6, extend the full length of the retina ($\sim 85\mu\text{m}$), and project to the first optic ganglion, the lamina (Rao, 2005). These photoreceptors all express the same blue/green sensitive rhodopsin (Rh1) (Scavarda et al., 1983; O'Tousa et al., 1985; Zuker et al., 1985) and they mediate motion vision (Rister et al., 2007; Yamaguchi et al., 2008). R7 and R8 localize in the center and are restricted to the upper and lower halves of each ommatidium, respectively, and they project to the second optic ganglion, the medulla (Rao, 2005). Each R7 or

R8 expresses only one opsin gene out of four central photoreceptor opsins that exhibit different spectral sensitivities, so they mediate color vision (Franceschini et al., 1981; Fryxell and Meyerowitz, 1987; Montell et al., 1987; Zuker et al., 1987; Chou et al., 1996; Huber et al., 1997; Chou et al., 1999).

Rhodopsin and arrestins

The light to electrical signal transduction (phototransduction) begins with rhodopsin, the light receptor (Fig.1.2). A rhodopsin consists of an opsin protein and a chromophore. Upon exposure to light, the chromophore (3-hydroxy-11-*cis* retinal in *Drosophila*) absorbs a photon and isomerizes almost instantaneously from 11-*cis* to all-*trans*. The isomerization alters the shape of the retinal, forcing a conformational change of the opsin, a G protein-coupled, seven transmembrane domain protein (Montell, 1999). The activated rhodopsin in turn activates the heterotrimeric G protein (Scott et al., 1995).

The deactivation of rhodopsin mainly depends on visual arrestins, which, competing with G protein, bind activated rhodopsin to deactivate it (Fig.1.2) (Ranganathan and Stevens, 1995). Arrestins comprise two domains of antiparallel β -sheets connected through a hinge region and one short α -helix on the back of the amino-terminal fold (Granzin et al., 1998). Both N-terminal and C-terminal domains of arrestin contribute in binding to rhodopsin (Skegro et al., 2007). There are two arrestins expressed in *Drosophila* photoreceptors, arrestin1 and arrestin2 (Arr1 and Arr2) (Smith et al., 1990; Hyde et al., 1990; Yamada et al., 1990; LeVine et al., 1990). Elimination of Arr2 significantly decreases the deactivation rate of photoresponse (Dolph et al., 1993). Although elimination of Arr1 does not show discernible delay in deactivation, the *arr1;arr2* double mutant displays a much slower deactivation rate compared to the *arr2* single mutant (Dolph et al., 1993), suggesting Arr1 also participates in deactivation. The dissociation of arrestins from rhodopsin may depend on the CaMKII-dependent phosphorylation of arrestins (Lu et al., 2009).

Besides being rapidly deactivated by visual arrestins, *Drosophila* rhodopsin also undergoes activity-dependent endocytosis for desensitization (Alloway et al., 2000; Kiselev et al., 2000; Satoh and Ready, 2005; Han et al., 2007). When exposed to light, three rhodopsin-interacting proteins (Arr1, Arr2 and G_q) bind to activated rhodopsin and induce rhodopsin endocytosis (Alloway et al., 2000; Kiselev et al., 2000; Satoh and Ready, 2005; Han et al., 2007). Arr1-mediated light-dependent rhodopsin endocytosis is detected at the pupal stage in wild-type flies (Satoh and Ready, 2005). Elimination of Arr1 blocks rhodopsin endocytosis and causes rhabdomeral degeneration, suggesting that Arr1-mediated endocytosis is critical for photoreceptor maintenance (Satoh and Ready, 2005). However, it is still unclear whether the endocytosed rhodopsin through the Arr1-mediated pathway is recycled back to rhabdomeral membrane for resensitization or is transported to lysosome for degradation. Arr2 forms stable complex with activated rhodopsin during light stimulation and recruits AP2 to induce rhodopsin endocytosis (Alloway et al., 2000; Kiselev et al., 2000; Orem et al., 2006). Excessive Arr2-mediated endocytosis leads to downregulation of rhodopsin (Orem and Dolph, 2002) and apoptotic photoreceptor cell death (Alloway et al., 2000; Kiselev et al., 2000). Although this endocytic pathway is only detected in mutant flies (Alloway et al., 2000; Kiselev et al., 2000; Satoh and Ready, 2005; Wang et al., 2005), it might also be critical for photoreceptor maintenance in wild-type flies because blockage of Arr2-mediated endocytosis results in rhabdomeral degeneration (Orem et al., 2006). G_q also induces light-dependent rhodopsin endocytosis in some mutant flies, including in *arr2* flies (Han et al., 2007). The consequence of excessive G_q -mediated endocytosis is to cause rhodopsin downregulation (Han et al., 2007). However, the importance of the G_q -mediated pathway is hard to evaluate because elimination of G_q in wild-type background does not have any morphological defect (Vinós et al., 1997). When light is off, rhodopsin is no longer being activated. Therefore, Arr1, Arr2 and G_q do not bind rhodopsin and endocy-

tosis does not occur. To my knowledge, there is no report to show the fate of internalized Arr1, Arr2 and G_q.

G protein

The effector of the light-activated rhodopsin is an eye-specific heterotrimeric G protein, G_q (Fig.1.2) (Scott et al., 1995). γ subunit of the $\beta\gamma$ dimer binds the activated rhodopsin (Kisselev and Downs, 2003). The α subunit binds GDP and associates with the $\beta\gamma$ dimer in the inactive state. Receptor activation accelerates the exchange of bound GDP for free GTP, followed by the dissociation of active G α -GTP from the $\beta\gamma$ subunits (Devary et al., 1987; Alberts et al., 2002). Unlike the mammalian visual G protein (G_t) that activates a phosphodiesterase (PDE) to close cGMP-gated channels (Squire et al., 2003), *Drosophila* G protein (G_q) stimulates a phospholipase C (PLC) (Bloomquist et al., 1988). At the same time, PLC functions as a GTPase-activating protein (GAP) to increase the rate of G α -binding GTP hydrolyzation, thereby deactivating G protein (Cook et al., 2000; Wang et al., 2008).

The α subunit (G _{α q}) of the heterotrimeric G protein undergoes light-dependent translocation, which is an important light adaptation mechanism of fly photoreceptor (Kosloff et al., 2003; Cronin et al., 2004; Elia et al., 2005; Frechter et al., 2007). During light stimulation, G _{α q} translocates from the rhabdomere to the cytosol within 5 min and the quantity of translocated G _{α q} depends on the intensity of illumination (Kosloff et al., 2003; Cronin et al., 2004). This process requires rhodopsin activity. Loss of rhodopsin causes G _{α q} to locate in the rhabdomere during light stimulation (Cronin et al., 2004) and constitutively active rhodopsin results in persistent localization of G _{α q} to the cytosol (Kosloff et al., 2003; Iakhine et al., 2004). After removal of light, G _{α q} gradually translocates from the cytosol to the rhabdomere, taking about 2.5 hours for full recovery (Kosloff et al., 2003; Cronin et al., 2004). The photoreceptor-specific myosin NINAC is required

for this process. The rate of $G_{\alpha q}$ transport from the cell body to the rhabdomere is significantly reduced in the *ninaC* null mutant (Cronin et al., 2004). The $\beta\gamma$ dimer is also required for the recovery of $G_{\alpha q}$ localization back to the rhabdomeral membrane. After removal of light, no recovery is detected in the $G\beta$ mutant (there is no detectable γ subunit in this $G\beta$ mutant by western blot) (Kosloff et al., 2003; Elia et al., 2005).

Phospholipase C and channels

$G\alpha$ -GTP activates PLC through binding its C-terminus (Kim et al., 1996), but the molecular details are unknown. Crystal structure of PLC illustrates occlusion of the active site by a loop separating the two halves of the catalytic TIM barrel (Hicks et al., 2008). However, it remains unclear how $G\alpha$ -GTP removes such autoinhibition.

Given that PLC catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Fig.1.2) (Alberts et al., 2002), there are two possibilities to open the light-sensitive channels, transient receptor potential (TRP) and transient receptor potential-like (TRPL) (Wong et al., 1989; Niemeyer et al., 1996; Reuss et al., 1997).

Several studies indicate that IP_3 is not involved in TRP and TRPL activation. Release of caged IP_3 is not capable of opening TRP and TRPL channels (Hardie and Raghu, 1998), and loss of the only known *Drosophila* IP_3 receptor in the eye has no effect on activation of TRP and TRPL (Acharya et al., 1997; Raghu et al., 2000a).

However, it appears that DAG or its metabolites are necessary for TRP and TRPL activation (Fig.1.2). TRP and TRPL are constitutively activated if DAG is not timely phosphorylated by DAG kinase (Raghu et al., 2000b). Although DAG usual effector, protein kinase C (PKC), is not required in this process, as loss of the eye-specific PKC does not affect the activation of phototransduction (Smith et al., 1991; Hardie et al., 1993); DAG may serve as a potential precursor of polyunsaturated fatty acids (PUFAs) to open TRP and

TRPL channels. Application of PUFAs from a nearby puffer pipette activates both TRP and TRPL channels (Chyb et al., 1999). Moreover, DAG lipase that catalyzes DAG to PUFAs is necessary for TRP channel activation (Leung et al., 2008).

The opening of TRP and TRPL channels leads to Ca^{2+} influx and depolarization of photoreceptors (Wong et al., 1989; Niemeyer et al., 1996; Reuss et al., 1997). The Ca^{2+} can be rapidly removed from photoreceptors by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger CalX during and after light stimulation (Wang et al., 2005a).

Photoreceptor output

Photoreceptor depolarization evokes the release of photoreceptor neurotransmitter histamine that gates chloride channels on large monopolar cells (LMCs), postsynaptic to photoreceptors in the lamina (Sarthy, 1991; Gengs et al., 2002; Pantazis et al., 2008). Adaptation improves the efficiency of neural encoding from photoreceptors to LMCs (Zheng et al., 2009; Nikolaev et al., 2009). Adaptation enhances both the frequency and amplitude distribution of LMC output by improving sensitivity to under-represented signals within seconds (Zheng et al., 2009). Moreover, such adaptation needs both the light-mediated conductance and feedback-mediated synaptic conductance (Nikolaev et al., 2009).

After light is off, the histamine in the synaptic cleft is removed by glial cells, where it is converted into carbinine (Fabian-Fine et al., 2003; Borycz et al., 2002). Carbinine is then released by glia and taken up by photoreceptor cells, where it is converted back into histamine (Borycz et al., 2002).

Drosophila phototransduction occurs in the rhabdomere, which is the analogue of the rod outer segment. A rhabdomere is made up of about 60,000 tightly packed microvilli (Fig. 1.1A) (Zuker, 1996). Like optic discs in the outer segment, these microvilli extensively increase the area of photosensitive membrane in the *Drosophila* photoreceptor. Additionally, more than 100 million rhodopsin molecules packed in the membrane of microvilli in each

rhabdomere (Zuker, 1996). Thus, similar to rod cells, *Drosophila* photoreceptors have a tightly organized photosensitive membrane structure and high rhodopsin concentration in the membrane, which together ensure a high amount of rhodopsin molecules in the sensory organelle and enable photoreceptors to achieve high sensitivities to light. Because of this, we can use *Drosophila* photoreceptor as a genetic model to study how it controls the activities of signaling molecules to prevent the sensory organelle from degenerating and how it maintains the high concentration of rhodopsin in the photoreceptive membrane.

1.3 Advantages of *Drosophila* in the study of visual functions

There are great technical advantages of using *Drosophila* to study visual functions.

First and foremost, *Drosophila* is a powerful model organism for genetic researches. Firstly, *Drosophila* genome is completely sequenced in 2000 (Adams et al., 2000; Myers et al., 2000). Much of *Drosophila* genome is evolutionarily conserved, and many of the genes found in *Drosophila* have orthologous human counterparts. In addition, the low level of genetic redundancy of *Drosophila* makes it easy to study the functions of these gene products. Secondly, a large number of advantageous techniques and tools are available to temporally and spatially manipulate gene expression in *Drosophila* (Johnston, 2002). Thirdly, *Drosophila* genetics is also greatly benefited by the shared resources from *Drosophila* stocks (Bloomington *Drosophila* Stock, Exelixis Collection, Vienna *Drosophila* RNAi Center et al.). Last but not least, *Drosophila* has a very short life cycle and one fly can produce up to a thousand progeny at minimal expense.

Additionally, defects in *Drosophila* visual functions can be detected by a simple assay, electroretinogram (ERG) recording. ERG records the summed potential change in the extracellular medium of the eye due to light-induced current flow. It mainly represents

the sum of the light-induced electrical activities in photoreceptors. Typical ERGs contain a negative plateau that is maintained throughout the light stimulus and transient spikes at both light-on and light-off, known as on-/off-transients (Fig. 1.3A). Upon light stimulation, the influx of cation through TRP/TRPL channels causes a rapid depolarization of photoreceptors and a decrease in the extracellular potential, which maintains during stimulation. After removing the light stimulation, TRP/TRPL channels close and ERG curve rapidly returns to the baseline. Most alterations of light response based on ERG recordings are due to defects in photoreceptors, although the on- and off-transients are believed to be caused by synaptic transmission between photoreceptors and their target neurons (Montell, 1999; Pak and Leung, 2003). If photoreceptors in a mutant fly have lower light sensitivities, we will need a higher light intensity to trigger a visible ERG response. Thus we can use ERG to easily screen a large number of flies to look for mutants with defective light response, including those with low visual sensitivities.

Moreover, the rhabdomere morphology of *Drosophila* can be examined in intact fly eyes using simple assays such as deep pseudopupil and optical neutralization assays. In the optical neutralization assay, fly heads are separated from the body and immersed in a layer of lens oil to optically neutralize the cornea. On the stage of a microscope, a spotlight is shone into the head from the neck side for antidromic illumination of the compound eye. The rhabdomeres that appear as bright dots due to high transmission of light are counted for each upright ommatidium (Franceschini and Kirschfeld, 1971). In this method, all six peripheral (R1-R6) and the R7 central rhabdomeres of each ommatidium are detected as individual light spots in wild-type flies (Fig. 1.3B). The light spot is invisible if the rhabdomere diminishes or has dissembled microvilli (Fig. 1.3B). Benefited from the optical neutralization assay, we can easily screen a large number of flies to look for mutants with defects in rhabdomeral morphology.

1.4 Maintenance of photoreceptor sensitivity in *Drosophila*

To reach high sensitivity, the *Drosophila* photoreceptor contains a high amount of rhodopsin, which is achieved by the tightly packed microvilli in the rhabdomere and the high rhodopsin concentration in microvillar membrane.

1) Maintenance of rhabdomeral structure

Photoreceptors appropriately regulate the activities of phototransduction signaling molecules to maintain rhabdomeral structure. Constitutive activities and improper regulation of visual signaling proteins always lead to necrotic photoreceptor degeneration (Ranganathan, 2003). Such degeneration can be protected by blockage of phototransduction. For example, the mutant of the major rhodopsin Rh1, *Rh1^{pp100}*, is constitutively activated and causes rhabdomeral degeneration, which can be partially rescued by genetic reduction of G_q level (Iakhine et al., 2004). Moreover, in *arr2* mutants, Rh1 cannot be timely deactivated, and thus induces light-dependent retinal degeneration (Dolph et al., 1993). The degeneration is suppressed by introducing a hypomorphic allele of *norpA* (which encodes PLC) (Alloway et al., 2000). The activity of TRP channel also needs to be appropriately regulated to prevent overstimulation-mediated necrotic photoreceptor degeneration. For example, the constitutive active mutant of TRP channel, *Trp^{P365}* shows severe degeneration and the degeneration is partially rescued by introducing a copy of hypomorphic allele of *trp* mutant, *trp^{CM}* (Yoon et al., 2000). Additionally, the *rdgA* mutant shows early on-set retinal degeneration. *rdgA* encodes a DAG kinase. Without DAG kinase, DAG cannot be phosphorylated and it will continually activate TRP channels. Genetic reduction of TRP level can suppress the *rdgA*-induced degeneration (Raghu et al., 2000b).

In addition to regulating the activities of signaling molecules, photoreceptors also tightly control the endocytosis of rhodopsin to prevent apoptotic degeneration. In *norpA* and *rdgC*

mutants, Arr2 cannot be dissociated from the inactivated form of Rh1 and thus forms a stable complex with Rh1, which leads to apoptotic photoreceptor degeneration (Alloway et al., 2000; Kiselev et al., 2000). The degeneration can be suppressed by introducing a mutant form of *Drosophila* dynamin, which is critical for most GPCR endocytosis. The degeneration can also be suppressed by blockage of the formation of the Rh1-Arr2 complex through reducing the expression level of Rh1 or Arr2 or mutating the Rh1-Arr2 binding sites in either molecule.

Similar mechanisms also function in mammalian photoreceptors. Appropriate regulation of rhodopsin signaling and binding affinity with visual arrestin plays a crucial role in the maintenance of outer segments in rod photoreceptors (Lem and Fain, 2004; Chen et al., 2006).

2) Maintenance of rhodopsin concentration

Although the high rhodopsin concentration in rhabdomeral membrane is obviously important for the maintenance of photoreceptor sensitivity, very limited studies have reported the mechanisms underlying the maintenance of rhodopsin concentration. Vitamin A is required for the production of rhodopsin. A lack of vitamin A in food leads to loss of night vision and this is primarily due to a lack of the high concentration of rhodopsin in light sensory organelles of photoreceptors in both mammals and *Drosophila* (Katz et al., 1991; Harris et al., 1977). Moreover, the visual arrestin Arr2 and the F box protein dFbx14 (regulated by dCAMTA) control the G_q-mediated Rh1 endocytosis to prevent the downregulation of Rh1 and the reduction of photoreceptor sensitivity (Han et al., 2007).

Previous studies have provided valuable information about the mechanisms to prevent reduction of photoreceptor sensitivity. There are still many important questions to be addressed. Two such questions are: How do photoreceptors regulate other signaling molecules (in addition to Rh1 and TRP) to maintain the structure of rhabdomeres? How do

photoreceptors antagonize the arrestin-mediated endocytosis to achieve the high rhodopsin concentration in rhabdomeral membrane? In this thesis, I present our findings regarding these questions.

In the second chapter of my thesis, I present a work that identifies TADR as a protein required to prevent G_q-mediated rhabdomeral degeneration. In the *tadr* mutant, activated G_q protein is retained on the rhabdomeral membrane, which causes rhabdomeral degeneration independent of PLC and leads to visual sensitivity reduction in an age-dependent manner (Fig. 1.4). These studies were published in *J Neurosci* (2008)28: 13478-13487.

In the third chapter of this thesis, I present a molecular mechanism that antagonizes Arr1-mediated rhodopsin endocytosis. Loss of a CUB- and LDLA-domain (CULD) protein leads to the Rh1 mislocalization and the reduction of photoreceptor sensitivity. CULD interacts with Arr1, which is important for the CULD *in vivo* function that counteracts Arr1-mediated Rh1 endocytosis, at least partially through recycling endocytosed Rh1 back to the rhabdomere.

In short, studies in this thesis have identified new mechanisms underlying the maintenance of visual sensitivity in *Drosophila*, either through preventing G_q-mediated rhabdomeral degeneration or through antagonizing rhodopsin endocytosis. This work advances our understanding of visual biology and the general regulatory mechanisms of GPCR signaling, and may provide valuable clues to pathologic studies of human retinal degeneration disorders, such as RP, which may help to reveal therapeutic approaches.

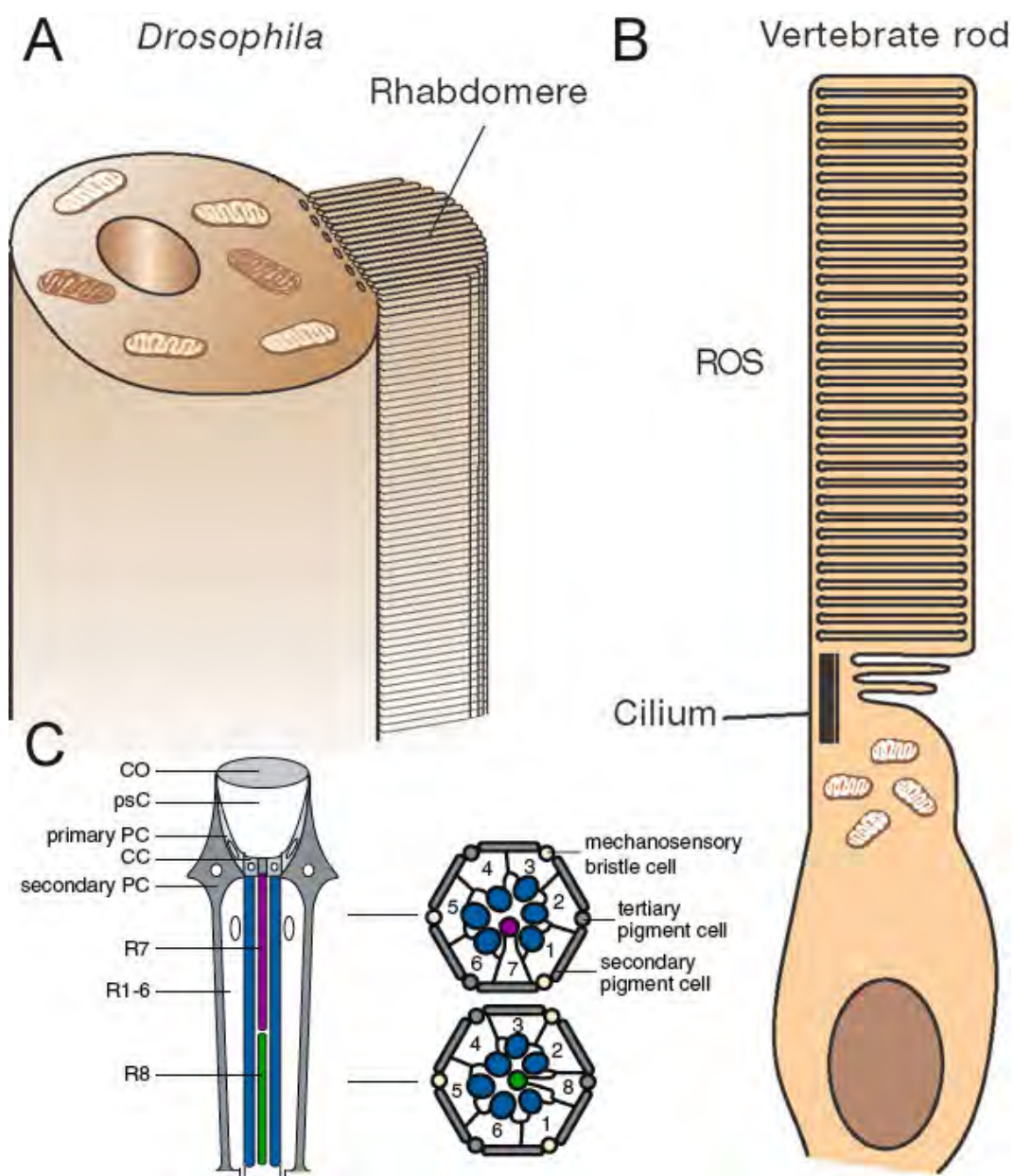


Figure 1.1

Figure 1.1. Photoreceptor structure.

A) In *Drosophila*, the photoreceptive membrane is organized into tightly packed, tubular microvilli, each 1–2 μm long and 60 nm in diameter, together forming a $\sim 100 \mu\text{m}$ -long rhabdomere.

B) Vertebrate rod outer segments (ROS) ($\sim 35 \mu\text{m}$ long) contain stacks of membranous discs ($\sim 1,000$), known as optic discs. In both cases the overall structure serves to maximize absorption of light by forming a cylindrical light-guiding structure with a high density of rhodopsin-containing membrane (Hardie and Raghu, 2001).

C) Single ommatidium. Left panel: single ommatidium at an angle 90° from the surface of the compound eye. co, cornea; primary PC, primary pigment cell; psC, pseudocone; secondary PC, secondary pigment cell; R1-6, photoreceptor cells 1-6; R7, photoreceptor cell 7; R8, photoreceptor cell 8. Right panel: cross sections through the distal and proximal regions of the ommatidium. Seven photoreceptor cells are present in any given plane of section. The ovals represent the rhabdomeres. The photoreceptor cell bodies are numbered. A secondary pigment cell, a tertiary pigment cell and a mechanosensory bristle cell are indicated (Wang and Montell, 2007).

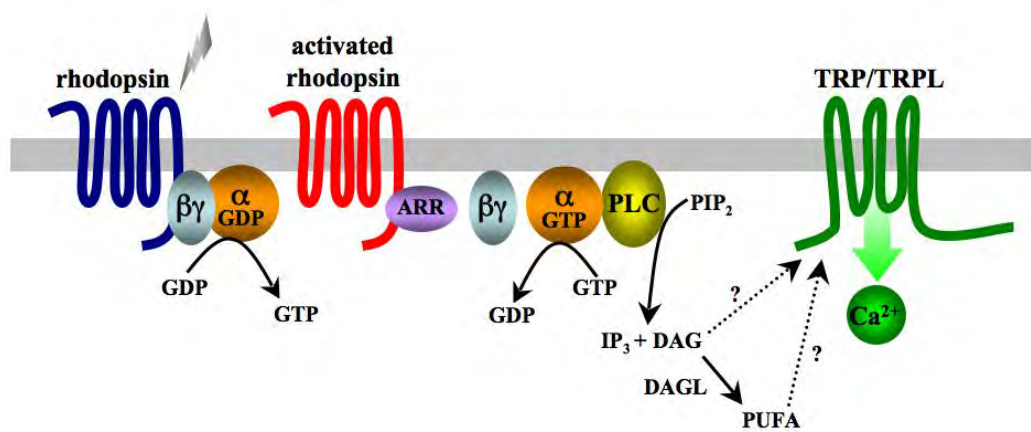


Figure 1.2

Figure 1.2. *Drosophila* phototransduction.

Upon light exposure, rhodopsin activates G protein, which in turn stimulates PLC. PLC hydrolyzes PIP_2 to IP_3 and DAG. DAG or its metabolites (PUFAs) are required for the opening of TRP and TRPL channels, thereby resulting in Ca^{2+} influx and photoreceptor depolarization. Visual arrestins bind activated rhodopsin to deactivate it and PLC functions as a GAP to deactivate G protein. See the text for detail.

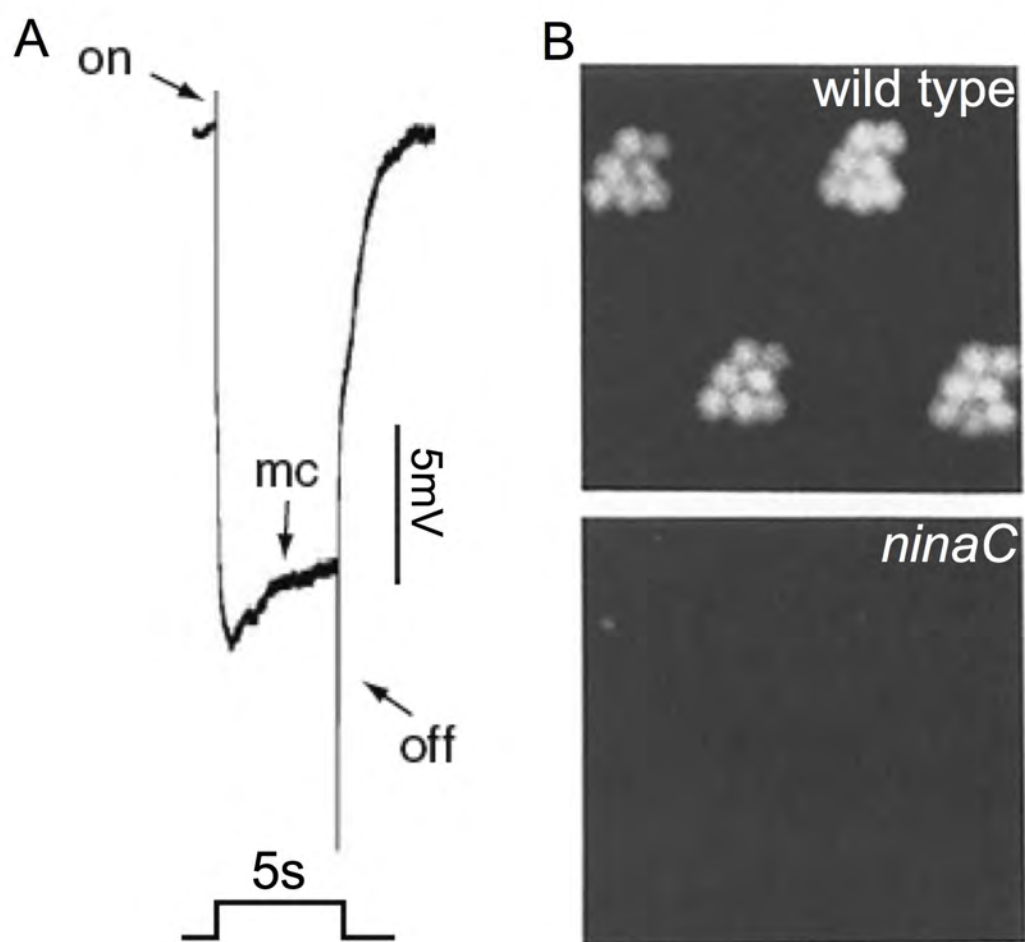


Figure 1.3

Figure 1.3. Electroretinogram (ERG) recordings and optical neutralization assay.

A) ERG performed on wild type using orange light. Amplitude (mV) and time-scale (s) markers are included. The on- and off-transients and the maintained component (mc) are indicated (Wang and Montell, 2007).

B) Light microscopic images of rhabdomeres of wild type (upper panel) and *ninaC^{P325}* (lower panel) visualized by the optical neutralization assay. Note that *ninaC^{P325}* rhabdomeres were severely degenerated (Porter and Montell, 1993).

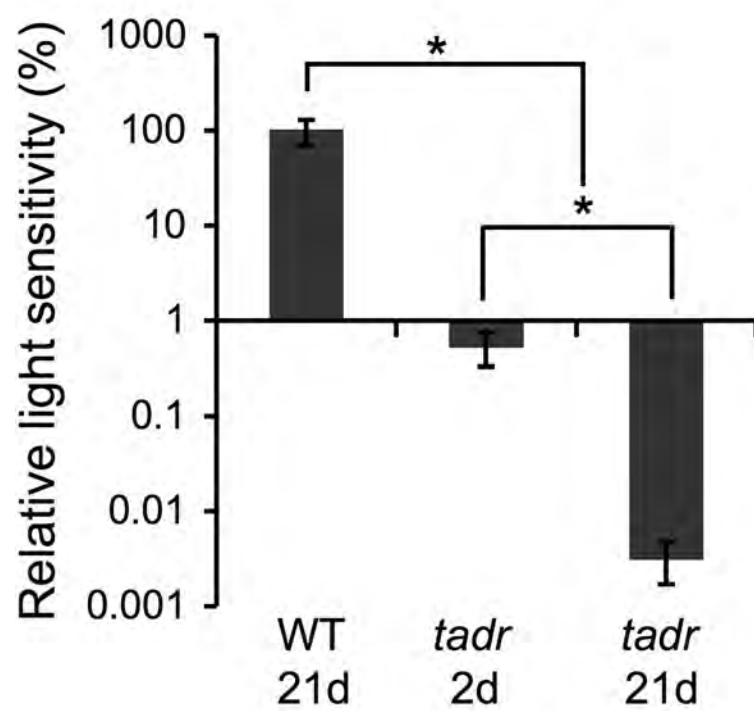


Figure 1.4

Figure 1.4. The *tadr* mutant shows an age-dependent visual sensitivity reduction. Flies were raised in normal light/dark cycles. The error bars represent s.e.m.. The asterisk (*) indicates the significant difference from the paired samples (*: $P < 0.05$).

CHAPTER II: MUTATION OF A TADR PROTEIN LEADS TO RHODOPSIN AND G_q-DEPENDENT RETINAL DEGENERATION IN *DROSOPHILA*

The work presented in this chapter is reproduced from a study by Ni et al., published in *J. Neurosci.* (Ni et al., 2008)

This work was conducted under the direction of Dr. Hong-Sheng Li and it is with gratitude to him and the other authors that I reproduce these data for the purpose of this dissertation. My contribution in this work was to execute the majority of the experiments including mapping the mutation, generating the transgenic fly and double mutants, optical neutralization analysis, toluidine blue staining, immunostaining, electroretinogram recordings, real-time RT-PCR, glutathione-sepharose binding assay, Arr2 binding and release assays, light-stimulated GTP γ S binding assay and light-dependent G_q localization assay. Peiyi Guo contributed by conducting the whole-cell recordings. Keith Reddig contributed by conducting electron microscopy and sectioning samples for toluidine blue staining. Mirna Mitra contributed by mapping the mutation. Dr. Hong-Sheng Li did the EMS screen. Dr. Hong-Sheng Li and I prepared the manuscript together.

2.1 Abstract

The *Drosophila* photoreceptor is a model system for genetic study of retinal degeneration. Many gene mutations cause fly photoreceptor degeneration, either due to excessive stimulation of the visual transduction (phototransduction) cascade, or through apoptotic pathways that in many cases involve a visual arrestin Arr2. Here we report a gene named *tadr* (for *t*orn *a*nd *d*iminished *r*habdomeres), which, when mutated, leads to photoreceptor degeneration through a different mechanism. Degeneration in the *tadr* mutant is characterized by shrunk and disrupted rhabdomeres, the light sensory organelles of photoreceptors. The TADR protein interacted *in vitro* with the major light receptor Rh1 rhodopsin, and genetic reduction of the Rh1 level suppressed the *tadr* mutation–caused degeneration, suggesting the degeneration is Rh1–dependent. Nonetheless, removal of phospholipase C (PLC), a key enzyme in phototransduction, and that of Arr2 failed to inhibit rhabdomeral degeneration in the *tadr* mutant background. Biochemical analyses revealed that, in the *tadr* mutant, the G_q protein of Rh1 is defective in dissociation from the membrane during light stimulation. Importantly, reduction of G_q level by introducing a hypomorphic allele of *G_{αq}* gene greatly inhibited the *tadr* degeneration phenotype. These results may suggest that loss of a potential TADR–Rh1 interaction leads to an abnormality in the G_q signaling, which in turn triggers rhabdomeral degeneration independent of the PLC phototransduction cascade. We propose that TADR–like proteins may also protect photoreceptors from degeneration in mammals including humans.

Key words: Retinal degeneration, Rhodopsin, G protein, Photoreceptor, *Drosophila*, GPCR, Cation amino acid transporter

2.2 Introduction

Degeneration of rod and/or cone photoreceptors is a defining characteristic of retinitis pigmentosa (RP), a subset of human hereditary retinal diseases that cause night blindness followed by progressive loss of vision (Hartong et al., 2006). Many identified causal genes of RP encode key components of the visual transduction (phototransduction) cascade in photoreceptors (Hartong et al., 2006; Daiger et al., 2007). For instance, mutation in the light receptor rhodopsin is a prevalent cause of autosomal dominant RP (Kaushal and Khorana, 1994; Wilson and Wensel, 2003), and loss of rhodopsin regulatory proteins, arrestins and a rhodopsin kinase, causes Oguchi disease, an autosomal recessive form of RP (Fuchs et al., 1995; Yamamoto et al., 1997; Dryja, 2000). In addition, several other RP genes are required for the trafficking and maturation of rhodopsin molecules (Hartong et al., 2006). Thus, abnormalities in rhodopsin signaling pathways are major causes of photoreceptor degeneration. Nonetheless, in many RP cases, it remains puzzling why the product of an affected gene is important for photoreceptor protection. More importantly, the mutant genes in about 40% of RP cases have yet to be identified (Hartong et al., 2006; Daiger et al., 2007).

The *Drosophila* photoreceptor is a genetic model system for the study of both phototransduction (Montell, 1999; Hardie and Raghu, 2001) and retinal degeneration (Ranganathan, 2003). The whole visual transduction cascade is localized in a packed microvillar structure rhabdomere (Hardie and Raghu, 2001), which is analogous to the outer segment of rod and cone photoreceptors. The fly rhodopsin is coupled to a G_q type G protein (Lee et al., 1994; Scott et al., 1995). Instead of activating phosphodiesterase (PDE) to close cGMP-gated channels as in mammalian photoreceptors, this fly visual G protein stimulates a *norpA* gene-encoded phospholipase C (PLC) to open TRP Ca^{2+} /cation channels

(Bloomquist et al., 1988; Hardie and Minke, 1992; Montell, 1999). To rapidly terminate the light response, the stimulated rhodopsin molecule is deactivated promptly through a visual arrestin Arr2 (Dolph et al., 1993) and a dCAMTA/dFbxl4 pathway (Han et al., 2006).

Similar to those in humans, fly mutations in phototransduction molecules including rhodopsin (Leonard et al., 1992; Kurada and O'Tousa, 1995; Iakhine et al., 2004), PLC (Meyertholen et al., 1987; Zinkl et al., 1990; Alloway et al., 2000), TRP (Hong et al., 2002; Wang et al., 2005) and arrestins (Dolph et al., 1993; Satoh et al., 2005) all cause age-dependent photoreceptor degenerations, which are generally characterized by diminished rhabdomeres. Several other visual proteins such as a diacylglycerol kinase RDGA and a rhodopsin phosphatase RDGC are also essential for photoreceptor protection (Masai et al., 1993; Kiselev et al., 2000). Fly photoreceptors may degenerate in a necrotic, Ca^{2+} -dependent manner due to prolonged stimulation of the phototransduction cascade, or through apoptotic processes (Wang and Montell, 2007). In several mutants including *rdgC* and *norpA*, rhodopsin forms a stable complex with Arr2 to trigger photoreceptor apoptosis (Alloway et al., 2000; Kiselev et al., 2000). Here we report the isolation of a mutant fly *tadr* that undergoes rhabdomeral degeneration through a new pathway.

2.3 Materials and Methods

Fly genetics

The genotype of wild-type flies is *cn,bw* unless mentioned otherwise in the text. The *tadr* mutant was generated from *cn* progenitors using the chemical mutagen ethyl methane-sulfonate (EMS), and recombined into a *cn,bw* background. Except for the dark-reared flies that were never exposed to light from the prepupal stage, all others were raised in an approximately 12 hr light (~250 lux) /12 hr dark cycle. The mutant alleles of other genes used in this work are *ninaE*⁵, *arr2*⁵, *norpA*²⁴, *G_{αq}*¹, and *glass*².

A wild-type *CG9264* cDNA was obtained through RT-PCR, subcloned into a pCaSpeR-*hs* vector, and injected into *w*¹¹¹⁸ flies to generate *p[hs-CG9264]* transgenic flies. The transgene was subsequently crossed into the *tadr* mutant background. To express the protein, flies were heat shocked for 1 hr at 37°C in a water bath once a day from late pupal stage and examined at 7-days old.

Optical neutralization analysis

This analysis was performed as described previously (Franceschini and Kirschfeld, 1971). In brief, fly heads were separated from the body and immersed in a layer of lens oil to optically neutralize the cornea. On the stage of a microscope, a spotlight was shone into the head from the neck side for antidromic illumination of the compound eye. The rhabdomeres that appeared as bright dots due to high transmission of light were counted for each upright ommatidium. The mean number of rhabdomeres per ommatidium was calculated for each genotype and condition based on the results of 30 ommatidia from 5 flies. Standard errors of means (SEMs) were presented as error bars in figures.

Toluidine blue staining and electron microscopy

Fly heads were hemisected, fixed with 2.5% glutaraldehyde in 50 mM sodium cacody-

late buffer on ice for 4 hours, washed with the buffer solution three times, and fixed again with 1% osmium tetroxide for 1 hour. After ethanol dehydration, the head tissues were embedded in LR White resin. Eye cross-sections were cut either at 1 μm thickness and stained with 1% toluidine blue for light microscopy, or at 100 nm for electron microscopy.

Immunostaining of Rh1

Hemisected fly heads were fixed with 4% of paraformaldehyde in PBS, dehydrated in acetone, and embedded in LR White resin. Eye cross-sections of 1 μm were cut and stained with a monoclonal Rh1 antibody (DSHB) and a FITC-conjugated secondary antibody.

Electrophysiological recordings

Electroretinograms were examined as previously described (Li and Montell, 2000) with minor modifications. Flies were immobilized with thin stripes of tape. Two glass micro-electrodes filled with Ringer's solution were put separately on the eye surface and the thorax (as reference). Five-second light pulses (2500 lux) were used to stimulate the eye after adapting the fly in the dark for one minute. The signal was amplified and recorded using a Warner IE210 Intracellular Electrometer. For the quantification of response amplitude, data from six flies were averaged and SEMs were calculated.

For whole-cell recordings, the ommatidia were isolated from flies eclosed within 2 hours in Ca^{2+} -free Ringer solution, and individual peripheral photoreceptors were recorded as described previously (Han et al., 2006). The pipette and bath solutions were (in mM) 100 potassium gluconate, 40 KCl, 2 MgCl_2 , 0.1 EGTA, 5 ATP, 0.5 GTP, 10 HEPES (pH 7.15) and 130 NaCl, 5 KCl, 1.8 CaCl_2 , 5 proline, 25 sucrose, 10 HEPES (pH 7.15), respectively. The resistance of recording pipettes was 5–6 $\text{M}\Omega$. Cells were clamped at -70 mV to examine light-induced currents.

Glutathione-sepharose binding assay

cDNA fragments encoding the intracellular loop IV and the C-terminal tail of TADR

were amplified through PCR and inserted into a pGEX-5X vector to express GST-fused proteins (GST-LOOP and GST-TAIL) in bacteria. The fusion proteins were purified with glutathione-sepharose beads (Amersham). The proteins on beads were incubated with wild-type fly head extracts in PBS that contains 0.2% Triton X-100 and protease inhibitors (Roche). After three washes with the incubation solution, the bound proteins were eluted and subjected to SDS-PAGE and Western blot. The Arr2 antibody is as described (Han et al., 2006) and the sources of other antibodies were Montell lab (TRP), DSHB (Rh1), and Sigma-Aldrich (G_q).

Arr2 binding and release assays

Arr2 binding assays were performed as previously described (Sato et al., 2005) with modifications. Five heads from dark-reared 1-day-old flies were added into a homogenization solution containing 250mM sucrose, 120mM KCl, 5mM $MgCl_2$, 1mM DTT, 10mM MOPS (pH7.0), and Complete protease inhibitors (Roche). For Arr2 binding, heads were exposed to bright blue light (700 lux) for 4 seconds, homogenized in the dark, and centrifuged at 13,000 g for 5 min to precipitate the membrane fraction. For release of membrane-bound Arr2, the blue light-treated heads were exposed to orange light for 8 seconds before homogenization and centrifugation. The pellet and supernatant fractions were separated under very dim red light and subjected to SDS-PAGE and Western blot.

Assay of light-stimulated $GTP\gamma S$ binding

One-day-old, dark-reared flies were divided into two groups of 25 flies. Fly heads of the dark group were collected under dim red light, homogenized in 125 μ l of the homogenization buffer, and centrifuged at 13,000 g for 5min to precipitate membrane. After one wash with the homogenization buffer, the membrane fraction was resuspended in 25 μ l of ice-cold reaction buffer (2mM 2-mercaptoethanol, 5mM $MgCl_2$, 5mM creatine phosphate, 50 U/ml creatine kinase, 0.25mM ATP, 15 μ M GDP, and 50mM Mops pH6.7). After

5 μ l was saved for the determination of G_q protein level using Western blot, the membrane sample was added into 80 μ l reaction buffer that contains 25nM $GTP\gamma^{35}S$, and incubated in the dark for 10 min at room temperature. Membrane sample of the light group was prepared and incubated in the same way, except that the incubation and all other steps were conducted under blue light illumination (700 lux). The reactions were terminated by addition of 0.5ml ice-cold rinsing solution (2mM 2-mercaptoethanol, 5mM $MgCl_2$, and 50mM Mops pH6.7) followed by prompt filtration through glass-fibre filters (Whatman). The filters were rinsed with 2.5 ml of solution for 4 times, and air dried. The radioactivity on each filter was measured using a Beckman liquid scintillation counter. The level of $GTP\gamma S$ binding in each sample was normalized to the G_q protein level. The light-stimulated binding was calculated by subtracting the value of dark group from that of light group.

Assay of light-dependent G_q localization

The light-dependent G_q localization was examined following a previously described method (Kosloff et al., 2003). One group of 6 dark-reared flies less than one day old were exposed to bright blue light (700 lux) for one hour, while another group were kept in the dark. The heads were removed under dim red light, homogenized in 30 μ l of hypotonic homogenization solution [20mM HEPES, pH 7.6, with protease inhibitors (Roche)], and centrifuged at 13,000 g for 5min to precipitate the membrane fraction. The pellet was washed, centrifuged again, and the supernatants were combined. Both the pellet and supernatant fractions were subjected to SDS-PAGE and Western blot.

2.4 Results

2.4.1 *tadr* flies undergo rhabdomeral degeneration

The *tadr* mutant was identified using an optical neutralization technique, which is for observation of light passing through each rhabdomere in the fly eye (Franceschini and Kirschfeld, 1971) (see method). In this method, all six peripheral (R1–R6) and the R7 central rhabdomeres of each ommatidium are detected as individual light spots in wild-type fly eyes (Fig. 2.1A). The light spot will be invisible if the rhabdomere diminishes or has dissembled microvilli. Based on this assay, we conducted a small-scale chemical mutagenesis screen for genes critical for rhabdomere integrity on chromosome 2. Out of 273 homozygote-viable lines, we isolated two fly mutants with undetectable rhabdomeres at the age of two weeks (Fig. 2.1A and data not shown). One mutant is a new allele of the gene *Pph13*, which is required for rhabdomere morphogenesis (Zelhof et al., 2003), the other is *tadr*.

To confirm that the invisibility of rhabdomere in the optical neutralization assay (Fig. 2.1A) is due to disruption of rhabdomere structure in the *tadr* mutant, we conducted electron microscopy (EM) to examine cross-eye sections of 2-week-old flies. In contrast to the tightly packed microvillar structure found in wild type, the mutant peripheral rhabdomeres had detached and broken microvilli (Fig. 2.1B), and the overall length of microvilli was much shorter than wild type. Based on this EM observation, we named the mutant *tadr*, for *torn and diminished rhabdomeres*. The central R7 rhabdomeres in the *tadr* mutant contained normally attached microvilli, although many of these rhabdomeres were deformed.

The *tadr* phenotype could either be caused by rhabdomeral degeneration, or due to a defect in the development of rhabdomeres. We examined eye sections of 1-day-old *tadr* mutant, and found that rhabdomeres were virtually normal in these young flies (Fig. 2.2A).

In contrast, the microvilli of most peripheral rhabdomeres were severely shortened in 7-day-old flies, although they were not detached as in 2-week-old flies (Fig. 2.2A). Thus, the *tadr* phenotype may reflect a degeneration of rhabdomeres. This is further supported by the following toluidine blue (TB) staining and optical neutralization assays.

The TB staining of cross-eye sections revealed the rhabdomeres of the wild-type fly as dark ovals, with peripheral ones much larger than the R7 (Fig. 2.2B). In *tadr* mutant flies, most rhabdomeres had normal morphology at 1 day old (Fig. 2.2B). When the fly became 7 days old, however, the average size of peripheral rhabdomeres severely decreased, with many of them even smaller than R7 (Fig. 2.2B,C). In optical neutralization analyses, most peripheral rhabdomeres were visible in 1-day-old flies, about half of them became invisible at 7 days, and by the 13th day, almost no peripheral rhabdomeres were detected (Fig. 2.2D and Fig. 2.9). Those rhabdomeres showing abnormal shapes and reduced sizes in the TB staining were probably not detectable in the optical neutralization assay due to their low capabilities of light transmission.

In EM analyses, no significant abnormality was observed in the cell bodies of peripheral photoreceptors at early stages of rhabdomeral degeneration (Fig. 2.2A), suggesting the degeneration is originated from the rhabdomeres. However, when the mutant fly grew to 2 weeks of age, many cell bodies also shrunk severely (Fig. 2.1B).

2.4.2 The *tadr* mutant has smaller light response

Despite the degeneration of rhabdomere, the peripheral photoreceptors in 7-day-old *tadr* mutant flies are responsive to light (Fig. 2.10). Nonetheless, in electroretinogram (ERG) recordings, the light responses of *tadr* fly had smaller amplitudes and slower termination when compared to wild type (Fig. 2.3A).

In 1-day-old *tadr* flies that only had a very subtle degeneration, the amplitude of ERG

response was already smaller than wild type and was the same as that in the more degenerated, 7-day-old mutant flies (Fig. 2.3A). This temporal discrepancy between the ERG defect and the degeneration phenotype may suggest that the small ERG response is not simply caused by the morphological abnormality in rhabdomere. However, we found these two phenotypes are genetically linked to each other, and thus could be due to mutation of the same gene.

2.4.3 *CG9264* is the gene disrupted in the *tadr* fly

As degeneration assays require long-time aging of flies, we instead mapped the *tadr* mutation based on the ERG phenotype. The ERG phenotype was uncovered by two deficiency chromosomes Df(2L)pr-A14 (missing a region from 37D2 to 39A4) and Df(2L)DS6 (missing 38F5 to 39E7), which located the mutation to the chromosome region 38F5–39A4. We further generated three small chromosomal deletions using FRT-containing piggyBac elements (Parks et al., 2004) and narrowed the mutant region to 39A1 (Fig. 2.3B). This region contains two predicted genes *CG9264* and *CG33511* and partially covers another two genes. By sequencing the genomic DNA, we identified a missense mutation in the gene *CG9264*, which changes the residue 532 Gly to Arg in the encoded product (Fig. 2.3B).

In quantitative RT-PCR analyses, the *CG9264* mRNA level was greatly reduced in fly heads of a *glass* mutant missing photoreceptor cells (Moses et al., 1989) (Fig. 2.11), suggesting a high expression level of *CG9264* in photoreceptors. To confirm that the *CG9264* mutation is responsible for the visual phenotypes, we generated a transgenic fly (*tadr*; *P[hs-*CG9264*]*) that expresses a wild-type *CG9264* cDNA in the *tadr* mutant background through a heat-shock promoter. According to both EM and TB staining assays, overexpression of this cDNA by heat shocking the flies once a day from late pupal stage virtually eliminated the rhabdomeral degeneration observed in 7-day-old *tadr* flies (Fig.

2.3C). In addition, ERG responses after heat shock in these transgenic flies became almost identical to wild type (Fig. 2.3D). Thus, *CG9264* is indeed the mutant gene in the *tadr* fly, and is subsequently referred to as *tadr*.

2.4.4 Rhodopsin mediates rhabdomeral degeneration in the *tadr* mutant

The *tadr* gene encodes a 634 amino acid protein that has 12 putative transmembrane segments, with the eleventh disrupted by the Gly⁵³² to Arg mutation in the mutant fly (Fig. 2.3B). The TADR protein does not contain any known protein domain or motif, except that the amino acid sequence is moderately homologous to several potential cation amino acid transporters (Verrey et al., 2004) including the human SLC7A4 (21% identical) and SLC7A1 (19% identical).

As the protein structure of TADR did not provide enough clues to the understanding of the *tadr* mutation-dependent rhabdomeral degeneration, we attempted to explain the degeneration by looking for known rhabdomeral proteins that interact with TADR. We fused the two largest cytosolic fragments of TADR separately to a glutathione-S-transferase (GST) protein, immobilized them to glutathione-sepharose beads, and used the beads to pull down proteins from fly head extracts. The result indicated that the intracellular loop IV of TADR but not its C-terminal tail specifically pulled down Rh1, the rhodopsin protein of peripheral rhabdomeres, from the head extracts (Fig. 2.4). Other examined visual proteins including G_q, PLC, TRP and Arr2 failed to interact with either TADR fragment. In control experiments, GST alone did not pull down any visual protein. These observations may suggest that the TADR protein, either directly or indirectly, associates with Rh1 in the photoreceptor.

Considering that loss of TADR-Rh1 interaction could cause an abnormal rhodopsin signaling event disruptive to rhabdomere, we investigated whether rhabdomeral degeneration

tion in the *tadr* mutant is stimulated by the activity of rhodopsin. We raised the mutant flies in a completely dark condition to prevent light activation of rhodopsin, and examined the rhabdomeres by TB staining at 7 days. Although some rhabdomeres still showed irregular shape and/or reduced size (Fig. 2.5A), the average size of peripheral rhabdomeres was only slightly smaller than wild type and was much larger than in light-exposed mutant flies (Fig. 2.5C). In addition, the light deprivation greatly reduced the speed of rhabdomere loss in optical neutralization assays (Fig. 2.5D). Thus, degeneration of rhabdomere in the *tadr* mutant is largely stimulated by light.

Like other G protein-coupled receptors, rhodopsin may have a low-level of spontaneous activity in the absence of light stimulation, as evident by quantum activation of the phototransduction cascade in the dark (Elia et al., 2005). We suspected that the mild degeneration of rhabdomere in dark-reared *tadr* flies might be stimulated by spontaneous rhodopsin activities, and that removal of the rhodopsin protein could have a greater effect on *tadr* phenotype rescue than light deprivation. To test this, we decreased the rhodopsin protein level by introducing a hypomorphic allele of *ninaE* (*ninaE*⁵), the gene encoding the Rh1 opsin, into the *tadr* mutant background. Although the *ninaE* single mutant itself may undergo retinal degeneration at a much later stage (Leonard et al., 1992), the shape and integrity of rhabdomere remained intact in 7-day-old flies (Fig. 2.5B,D), except that the size of each peripheral rhabdomere appeared smaller than wild type (Fig. 2.5B,C). According to TB staining assays, the rhabdomeres in *tadr;ninaE* double mutant flies had the same size and shape as in the *ninaE* single mutant at 7 days (Fig. 2.5B). The average sizes of peripheral rhabdomere in both flies are significantly larger than that in the *tadr* single mutant. Moreover, the double mutant flies did not show any significant rhabdomeral loss in optical neutralization assays at least within 13 days after eclosion (Fig. 2.5D). Thus, decrease of Rh1 level suppresses the *tadr* mutation-caused rhabdomeral degeneration. Al-

together, these observations suggest that the *tadr* degeneration is mediated by the activity of rhodopsin.

2.4.5 Arr2 is not required for the rhabdomeral degeneration in *tadr* fly

Activated Rh1 rhodopsin forms a stable complex with a visual arrestin Arr2 to trigger apoptotic photoreceptor degeneration in several mutant flies (Alloway et al., 2000; Kiselev et al., 2000). However, such an apoptotic mechanism may not underlie the rhabdomeral degeneration in the *tadr* mutant. First, the blue light-generated Rh1–Arr2 complex in the *tadr* fly disassembled upon exposure to orange light as in wild type (Fig. 2.6A), indicating the lack of a stable complex in the mutant. Second, in both 1-day-old (Fig. 2.6B) and 10-day-old (Fig. 2.12) *tadr* flies, we failed to detect massive endocytosis of Rh1, which is required for the Rh1–Arr2 complex to trigger retinal degeneration (Orem and Dolph, 2002).

To further investigate whether Arr2 is involved in the *tadr* rhabdomeral degeneration at all, we examined the effect of *tadr* mutation in an *arr2* null background. In regular, cyclic illuminating conditions, although *arr2* single mutant flies had reduced size of rhabdomere and large intracellular vacuoles in the photoreceptor (Fig. 2.6C,D) due to necrotic degeneration (Alloway et al., 2000), the rhabdomere shape was in general normal at 7 days according to TB staining assays (Fig. 2.6C). In contrast, in *tadr;arr2* double mutant flies of the same age, many rhabdomeres either had irregular shape or completely disappeared (Fig. 2.6C). The average size of peripheral rhabdomeres was much smaller than that of the *arr2* single mutant (Fig. 2.6D). Moreover, in optical neutralization assays, the number of visible rhabdomeres in the double mutant decreased at a speed similar to that in the *tadr* single mutant, which is much faster than in the *arr2* mutant. (Fig. 2.6E). Thus, removal of Arr2 does not suppress the *tadr* rhabdomeral degeneration, suggesting that Rh1 mediates degeneration independent of Arr2 in the *tadr* mutant.

2.4.6 The *tadr* rhabdomeral degeneration is not caused by overstimulation of the phototransduction cascade

In the phototransduction cascade, rhodopsin stimulates Ca^{2+} -permeable TRP channels by the mediation of G_q and PLC. As excessive Ca^{2+} influx through TRP may cause necrotic damage to the photoreceptor (Raghu et al., 2000b), it is possible that the *tadr* rhabdomeral degeneration is due to prolonged or excessive stimulation of TRP channels by rhodopsin. However, electrophysiological recordings of peripheral photoreceptor isolated from newly eclosed flies do not support this hypothesis. First, in the dark, *tadr* mutant photoreceptors had background inward currents as small as wild type (Fig. 2.7A) and a reversal potential similar to wild type (-54.4 ± 5.0 vs -51.8 ± 4.5 mV). These observations indicate a lack of TRP channel activity in the dark and thus cannot explain the moderate, light-independent degeneration. Second, consistent with the ERG phenotype, the light response of *tadr* photoreceptor had a smaller instead of a larger amplitude compared to wild type (Fig. 2.7B), indicating a lower level of TRP stimulation by light.

A PLC mutation *norpA* prevents rhodopsin from stimulating TRP channels (Bloomquist et al., 1988). To further test whether the *tadr* rhabdomeral degeneration depends on TRP activity or any other PLC-mediated signaling event, we generated a *norpA;tadr* double mutant. When *norpA* single mutant flies were raised in cyclic illumination conditions to 7 days old, the peripheral rhabdomeres were only slightly different from wild type in shape, and were significantly larger than those in the *tadr* mutant (Fig. 2.7C,D). In contrast, the rhabdomeres in the *norpA;tadr* double mutant diminished more severely than in the *tadr* mutant (Fig. 2.7C,D). Additionally, the optical neutralization assay showed that the speed of rhabdomere loss in the double mutant was similar to that of *tadr* mutant fly, and was initially much faster than seen in the *norpA* single mutant (Fig. 2.7E). These observations suggest that rhodopsin mediates rhabdomeral degeneration independent of PLC and TRP

activities in the *tadr* mutant.

2.4.7 Abnormal G_q signaling may trigger rhabdomeral degeneration in the *tadr* mutant

The small amplitude of light response suggests that a phototransduction step is impaired in *tadr* photoreceptors. If the abnormality at this step also leads to the rhabdomeral degeneration, it should occur at the level of rhodopsin or G_q , because the degeneration is independent of the downstream molecules PLC and TRP. To help to understand the degeneration mechanism, we examined whether the step of G_q activation is impaired in the *tadr* photoreceptor. We prepared membrane samples from fly heads and measured blue light-stimulated $GTP\gamma S$ binding of membrane. Surprisingly, the level of stimulated $GTP\gamma S$ binding in the *tadr* membrane sample was even higher than in wild type (Fig. 2.8A).

To find out why light stimulated more $GTP\gamma S$ binding to the mutant membrane, we examined the effect of light stimulation on the level of membrane-associated $G_{\alpha q}$ molecules in the *tadr* mutant. As an important light adaptation mechanism of the fly photoreceptor, a large fraction of active $G_{\alpha q}$ molecules dissociates from membrane through depalmitoylation and diffuse out of the rhabdomere in bright light conditions (Kosloff et al., 2003; Cronin et al., 2004; Frechter et al., 2007). We found that, compared to wild type, a much lower amount of $G_{\alpha q}$ protein in the *tadr* mutant dissociated from membrane during light stimulation (Fig. 2.8B), which could partially explain the higher level of $GTP\gamma S$ binding to the mutant membrane. As the higher density of active $G_{\alpha q}$ protein on the mutant membrane leads to a weaker but not a stronger activity of TRP channel, the above observations might suggest that $G_{\alpha q}$ molecules in the *tadr* mutant are suffering from a problem, such as a low mobility on the membrane, which keeps them in the vicinities of rhodopsin molecules and restrains them both from depalmitoylation and from stimulating PLC.

Considering that prolonged possessing active $G_{\alpha q}$ molecules in a small membrane region could be harmful to the membrane structure, we examined the effect of decreasing $G_{\alpha q}$ level on the *tadr* rhabdomeral degeneration. A hypomorphic mutation ($G_{\alpha q}^1$) of the $G_{\alpha q}$ gene reduces $G_{\alpha q}$ to an undetectable level (Scott et al., 1995) without causing obvious morphological change in rhabdomeres of 7-day-old flies (Fig. 2.8C). According to TB staining assays, the *tadr* mutation failed to induce significant rhabdomeral degeneration in this $G_{\alpha q}^1$ mutant background at the age of 7 days (Fig. 2.8C,D). Moreover, in optical neutralization assays, the *tadr*-dependent rhabdomere loss was greatly inhibited in the *tadr*; $G_{\alpha q}$ double mutant (Fig. 2.8E). Thus, an abnormal $G_{\alpha q}$ signaling event may mediate the rhodopsin-dependent rhabdomeral degeneration in the *tadr* mutant.

2.5 Discussion

We have identified TADR as a potential Rh1–interacting protein that is essential for the protection of fly photoreceptor from degeneration. In the *tadr* mutant fly, rhabdomeres undergo rhodopsin activity–dependent degeneration, which is mediated by G_q through a pathway different from the phototransduction cascade.

TADR is homologous to human membrane proteins SLC7A4 and SLC7A1, which belong to the family of cation amino acid transporters (Verrey et al., 2004). Nonetheless, not all members of this family function as amino acid transporters. For example, SLC7A4 could not transport any amino acid into the cell after being expressed on the membrane of *Xenopus* oocytes (Wolf et al., 2002). These TADR/SLC7A proteins may have functions in addition to amino acid transport. In the *tadr* mutant, the rhabdomeral degeneration is not likely due to a shortage of amino acid supply in the photoreceptor. First, we did not detect a general problem in the synthesis of visual signaling proteins in western blot assays (L. Ni and H.–S. Li, unpublished observations). Second, if the rhabdomeral degeneration were due to the lack of particular amino acids, it should not be suppressed specifically by reducing the level of rhodopsin and G_q . It is more likely that loss of a different TADR function has caused the degeneration.

Our biochemical data indicates that TADR may interact, either directly or indirectly, with the Rh1 rhodopsin through the intracellular loop IV. In the *tadr* mutant, the G⁵³²R mutation disrupts the 11th transmembrane domain, resulting in an extended loop V that could block the Rh1–interacting site in the neighboring loop IV. As the TADR–Rh1 interaction may regulate Rh1–triggered signaling, loss of this interaction could lead to the Rh1–dependent rhabdomeral degeneration seen in the *tadr* mutant.

Abnormal signaling activities of rhodopsin may cause retinal degeneration through both

apoptotic and necrotic pathways in the fly eye. In several mutant flies, including *rdgC* and *norpA*, activated Rh1 rhodopsin forms a stable complex with Arr2 to trigger apoptotic photoreceptor degeneration (Alloway et al., 2000; Kiselev et al., 2000). This apoptotic pathway does not underlie the rhabdomeral degeneration in the *tadr* mutant, because the degeneration depends on the G_q protein instead of Arr2. By the mediation of the PLC NorpA, G_q could stimulate excessive Ca^{2+} influx through TRP channels, which leads to necrotic degeneration of photoreceptor (Dolph et al., 1993). Nonetheless, this Ca^{2+} -dependent necrosis is not responsible for the *tadr* rhabdomeral degeneration: first, the degeneration is independent of NorpA; second, the TRP activity is even lower in the *tadr* mutant. A different mechanism is likely underlying this G_q -dependent degeneration.

G_q also mediates PLC/TRP-independent photoreceptor degeneration in a dominant *ni-naE* mutant (Iakhine et al., 2004), although the mechanism remains unknown. After light stimulation, many more $G_{\alpha q}$ molecules in the *tadr* mutant are retained on the membrane compared to wild type. The lower level of phototransduction might suggest that most of the active $G_{\alpha q}$ molecules on membrane have failed to stimulate PLC in the mutant. We hypothesize that those extra $G_{\alpha q}$ molecules may instead have recruited an alternative effector to the membrane, which leads to the rhabdomeral degeneration. In addition to PLC, the G_q family proteins stimulate several other enzymes including an ADP-ribosylation factor ARF6 (Giguère et al., 2006) and a Rho guanine nucleotide exchange factor p63RhoGEF (Lutz et al., 2005; Lutz et al., 2007; Rojas et al., 2007). Both ARF6 and Rho are monomeric GTPases that may change morphology of membrane structures by modulating the underneath actin cytoskeleton (D'Souza-Schorey and Chavrier, 2006; Linseman and Loucks, 2008). More importantly, Rho GTPases have been found to mediate both apoptotic and necrotic pathways of neuronal death (Linseman and Loucks, 2008). In the future it would be interesting to investigate whether any fly ARF or Rho GTPase signals downstream of G_q in the

tadr rhabdomeral degeneration.

Another question remains to be addressed is exactly how the TADR protein helps to prevent the abnormal G_q protein signaling observed in the mutant. We propose that TADR may facilitate the dissociation of G_q protein from rhodopsin during light stimulation. In wild-type flies, TADR may form a complex with rhodopsin and the heterotrimeric G_q protein in dark condition. Upon light exposure, rhodopsin is conformationally changed, which not only activates G_q protein but also may activate TADR so that TADR could facilitate the dissociation of G_q protein from rhodopsin. The activated, rhodopsin-free G_q protein in turn activates PLC, which is required for the opening of TRP and TRPL channels. In the *tadr* mutant, however, the interaction between rhodopsin and TADR may be disrupted and thus activated G_q protein could not be efficiently dissociated from rhodopsin to stimulate PLC. Thereby light stimulates a higher level of $GTP\gamma S$ binding to membrane but still causes a lower degree of photoreceptor depolarization in the *tadr* mutant.

The mammalian visual G protein transducin also mediates degeneration of the rod photoreceptor cell. In mouse mutants that have prolonged rhodopsin activities, rod photoreceptors undergo transducin-dependent apoptosis upon exposure to low-intensity light (Hao et al., 2002). Thus, visual G proteins may play a pivotal role in the degeneration of photoreceptor.

We propose that some mammalian TADR-like proteins could control the activities of rhodopsin/transducin and help to prevent degeneration of rod and cone photoreceptors. Although it has not been reported that a SLC7A protein exists in the mammalian eye, several transporter proteins that contain twelve transmembrane domains like TADR are highly expressed in the mouse retina (Blackshaw et al., 2001). One such protein, a taurine transporter, has been demonstrated to be essential for retinal protection using a knockout mouse (Heller-Stilb et al., 2002; Rascher et al., 2004). It would be interesting to examine

whether any of these mammalian retinal transporter proteins interact with rhodopsin and/or regulate the signaling of transducin. To identify additional affected genes in human RP, those encoding multiple–transmembrane–domain proteins in the super family of “amino acid transporter” should be evaluated as candidates of a high priority.

2.6 Acknowledgments

We thank Dr. Craig Montell for the TRP antibody, and Dr. Charles S. Zuker for the $G_{\alpha q}^1$, Dr. Patrick J. Dolph for the *arr2*⁵, and the Harvard Exelixis Collection for the piggyBac insertion flies. We thank Jianwu Bai and people in the Li lab for discussions and for critical comments on the manuscript. This work was supported by a NIH grant R01–AG022508 awarded to H. L.

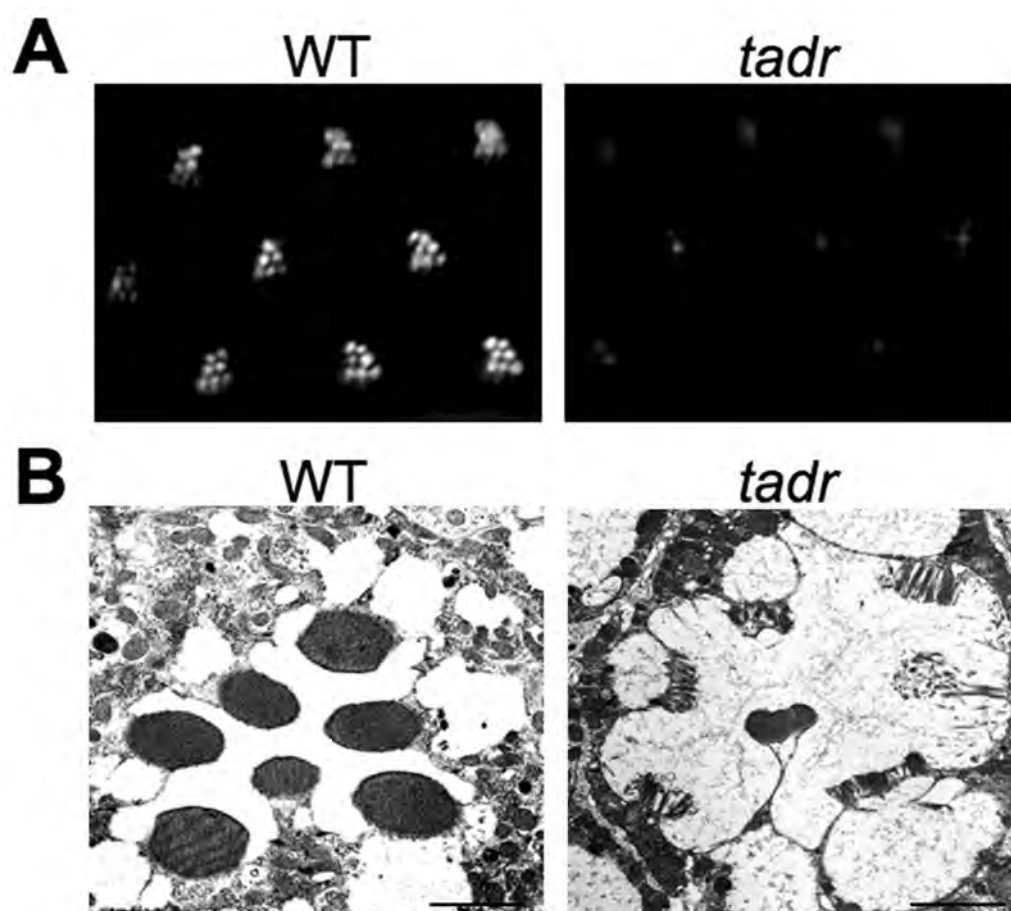


Figure 2.1

Figure 2.1. Impaired rhabdomere structure in *tadr* mutant flies.

A, A severe loss of peripheral rhabdomeres in 2-week-old *tadr* flies was observed in the optical neutralization assay.

B, EM analyses revealed that 2-week-old *tadr* flies contained detached and broken microvilli in peripheral rhabdomeres. Each picture shows a single ommatidium. Scale bars: $2\mu\text{m}$.

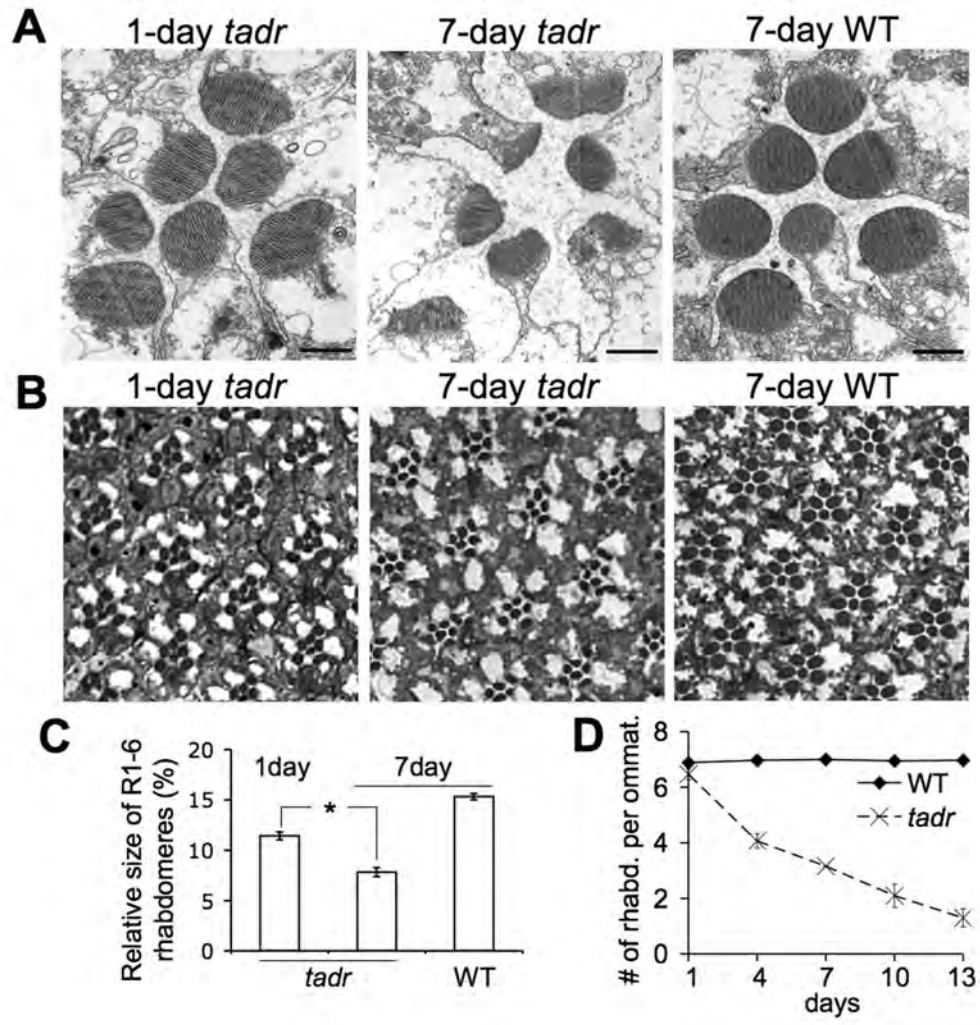


Figure 2.2

Figure 2.2. The rhabdomeral defect in the *tadr* mutant is caused by degeneration.

A, EM pictures for the comparison of rhabdomere structure between 1- and 7-day-old *tadr* flies. A wild-type picture is shown on the right. Scale bars: $1\mu\text{m}$.

B, TB staining of eye cross-sections revealed that 1-day-old *tadr* flies contained larger peripheral rhabdomeres than 7-day-old flies. Note the irregular shapes of some rhabdomeres in the 7-day mutant flies.

C, Quantification of the peripheral rhabdomere size based on the TB staining. The relative size represents the average ratio of the area occupied by all peripheral rhabdomeres to the total ommatidium area. Each ratio was calculated based on 6 ommatidia in a single eye section. Data from three experiments were averaged. SEMs are shown as error bars. The asterisk (*) indicates a significant difference.

D, In optical neutralization assays, the number of visible rhabdomeres decreased gradually during the aging of *tadr* flies. The mean number of rhabdomeres (rhabd.) per ommatidium (ommat.) was calculated based on 30 ommatidia of 5 flies for each genotype and age. Error bars represent SEMs.

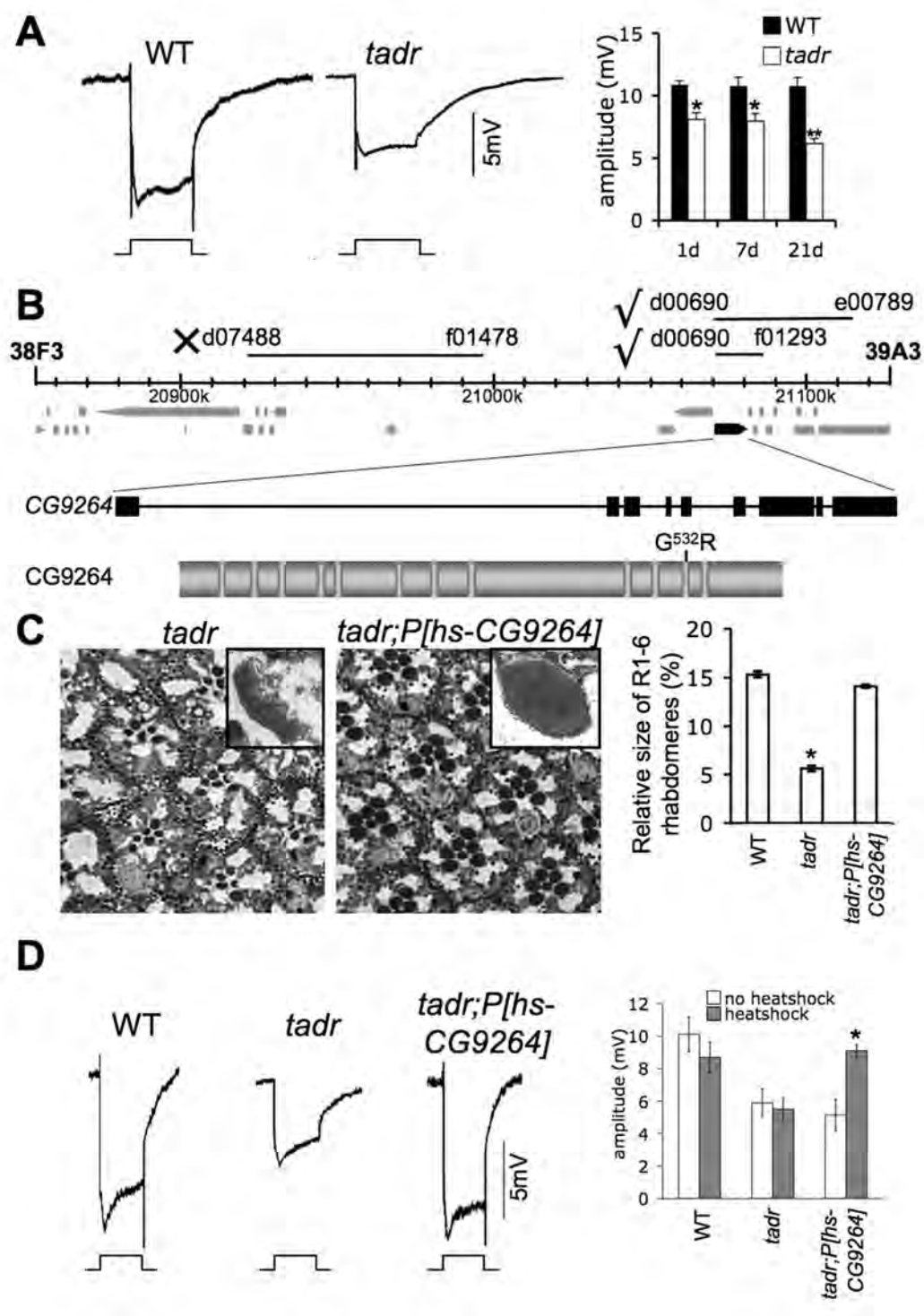


Figure 2.3

Figure 2.3. Identification of the *tadr* mutant gene.

A, ERG recordings revealed a small light response phenotype in the *tadr* mutant. Sample traces of ERG response in 7-day-old flies are shown on the left. The event markers underneath represent 5s orange light pulses. The right panel shows the amplitudes of ERG response at different ages. The asterisk (*) indicates significant differences from the wild-type controls.

B, The *tadr* mutation was mapped to the gene *CG9264*. Three chromosomal regions (top) between the shown piggyBac insertion sites were deleted separately using a flipase. Deletion of the right two regions, not the left one, uncovered the *tadr* ERG phenotype in complementation tests. A missense mutation was identified in the gene *CG9264*, which encodes a 12-transmembrane-domain protein (bottom).

C, After being expressed through a heat-shock promoter, a wild-type *CG9264* cDNA prevented rhabdomeral degeneration in 7-day-old *tadr;P[hs-*CG9264*]* transgenic flies. TB staining and EM (inset) results were shown on the left. The relative sizes of peripheral rhabdomeres were calculated based on the TB staining and shown in the right panel. The asterisk (*) indicates significant differences from the wild-type controls.

D, The *tadr* ERG phenotype was also rescued by overexpression of the *CG9264* cDNA. The sample ERG traces on the left are from heat-shocked flies. All recorded flies had a *cn* background. The asterisk (*) indicates significant differences from the no heat-shocked *tadr;P[hs-*CG9264*]* flies.

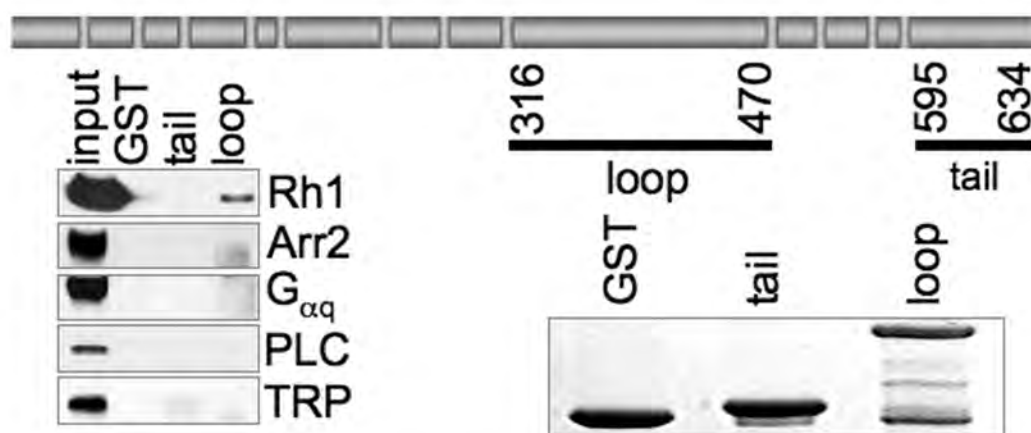


Figure 2.4

Figure 2.4. Rh1 interacts with TADR protein *in vitro*.

A GST-fused intracellular loop IV of TADR specifically pulled down Rh1 rhodopsin from fly head extracts in a glutathione-sepharose binding assay. The tail of TADR did not bind to any visual protein. Lane one was loaded with 1/15 of extract input. The coomassie-staining gel on the lower right shows the protein levels of GST and the GST-fusion proteins in the reaction mixtures.

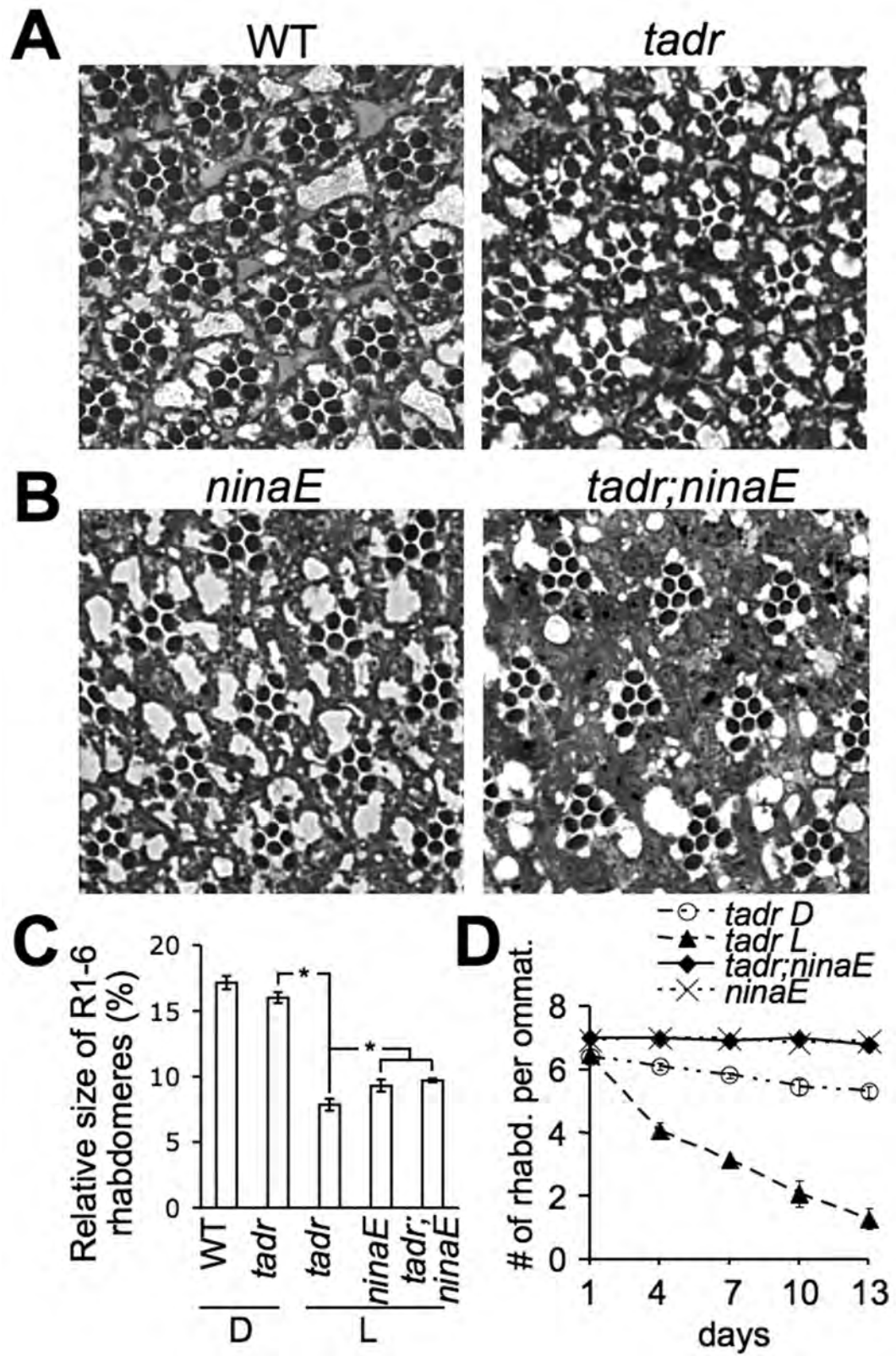


Figure 2.5

Figure 2.5. The *tadr* rhabdomeral degeneration depends on the rhodopsin activity.

A, Dark-reared *tadr* flies only showed a subtle rhabdomeral degeneration. Both the wild type and the mutant were dark-reared and 7 days of age.

B, Decreasing Rh1 rhodopsin level by a hypomorphic *ninaE* mutation prevented *tadr* mutation-caused rhabdomeral degeneration.

C, Relative sizes of peripheral rhabdomeres in different flies and light conditions. D: dark reared; L: raised in a normal light/dark cycle. The asterisk (*) indicates significant differences between the paired samples.

D, Optical neutralization assays showed that both light deprivation and reduction of Rh1 level prevent the severe rhabdomere loss caused by *tadr* mutation.

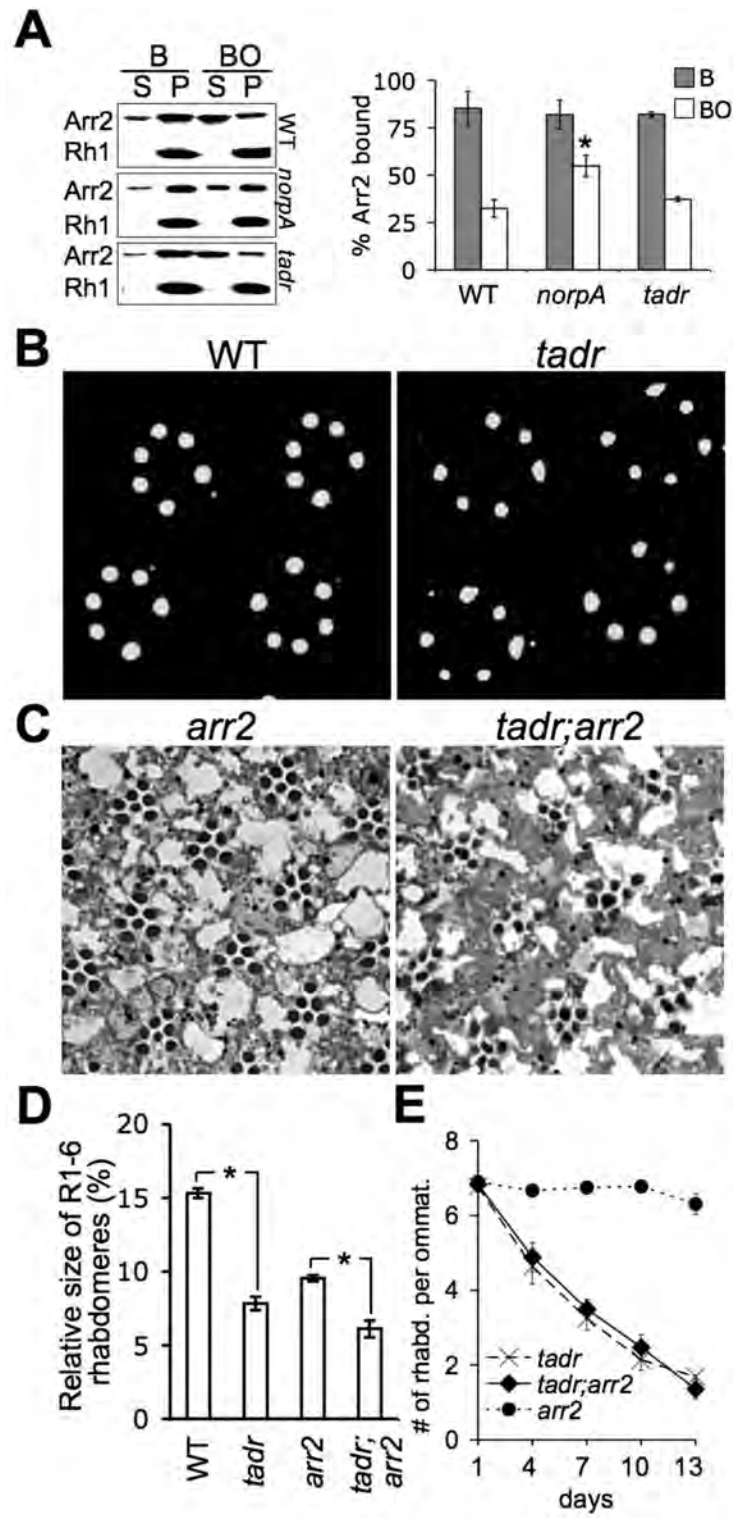


Figure 2.6

Figure 2.6. The Rh1–Arr2 complex is not involved in the *tadr* rhabdomeral degeneration.

A, Blue light–triggered binding between Arr2 and Rh1 was reversed by subsequent exposure to orange light in both wild-type and *tadr* mutant flies, but not in *norpA* mutant flies. All fly heads were collected in the dark. One group was exposed to blue light (B) and the other group was exposed to blue and then orange light (BO), before the homogenization. Supernatant (S) and membrane pellet (P) fractions were subjected to Western blot. The percentages of Arr2 bound to Rh1–containing membranes were quantified using a NIH ImageJ software. The averaged data of four independent experiments is shown in the right panel. The asterisk (*) indicates significant differences from the wild-type controls.

B, Immunostainings showed that the majority of Rh1 protein in 1–day–old *tadr* flies was localized in the peripheral rhabdomeres (the six large spots of each ommatidium). The number of small Rh1 endocytic particles in the cell–body areas was as low as in wild type.

C, Micrographs of TB staining showing more severe rhabdomeral degeneration in the *tadr;arr2* fly than in the *arr2* single mutant at 7 days.

D, Relative sizes of peripheral rhabdomeres measured in TB staining assays. The asterisk (*) indicates significant differences between the paired samples.

E, In optical neutralization assays, loss of visible rhabdomeres in the *tadr;arr2* fly was as rapid as in the *tadr* single mutant.

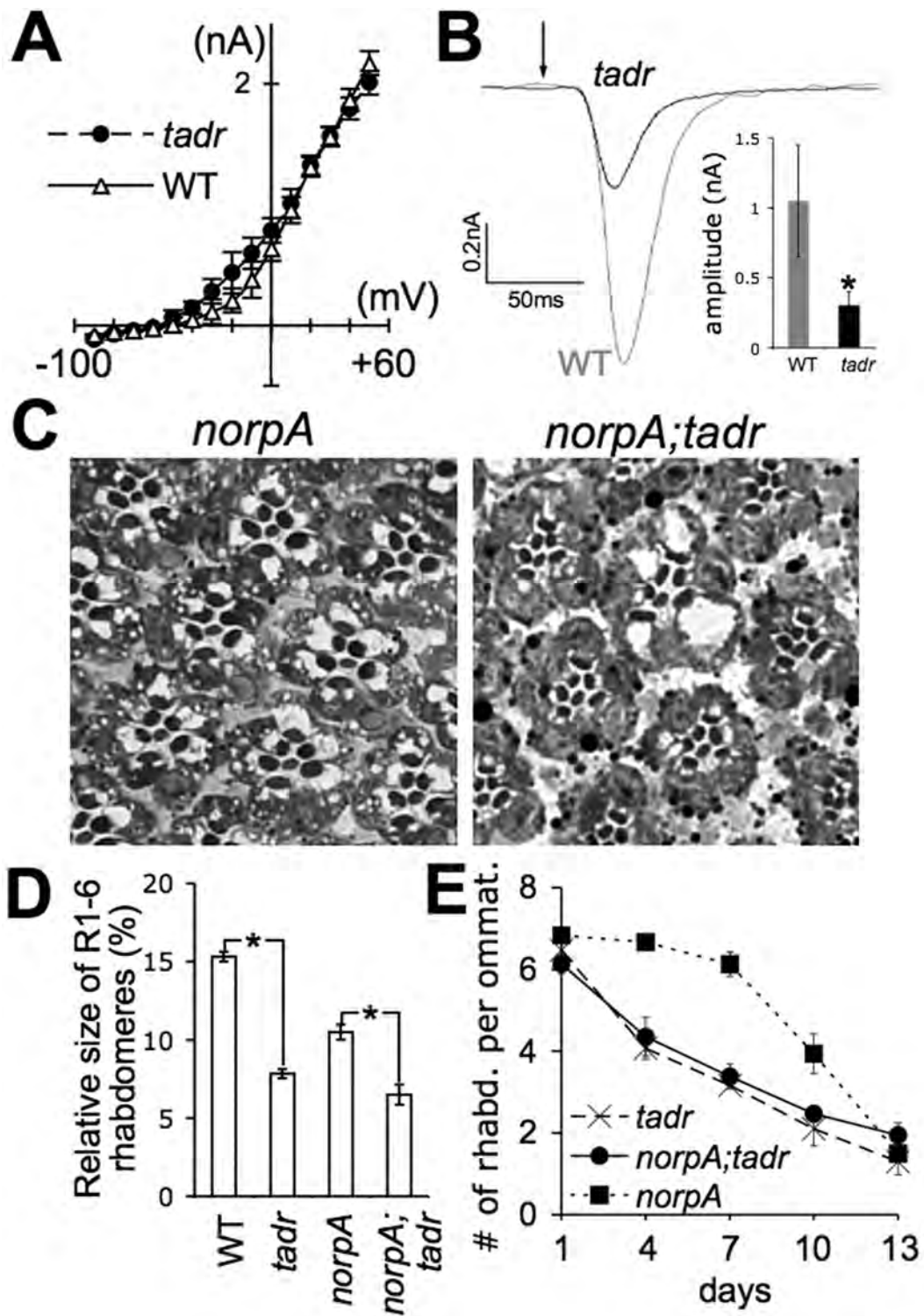


Figure 2.7

Figure 2.7. The *tadr* rhabdomeral degeneration is not caused by overstimulation of the phototransduction cascade.

A, In whole-cell current recordings, *tadr* mutant photoreceptors displayed normal background currents at different voltage levels in the dark. Data from three experiments were averaged.

B, A 10 ms light flash stimulated a smaller inward current in *tadr* mutant photoreceptors compared to that in wild type. The cells were clamped at -70mV in whole-cell configuration. The averaged amplitudes are shown on the lower right. The asterisk (*) indicates significant differences from the wild-type controls.

C, According to TB staining, loss of the PLC NorpA did not inhibit rhabdomeral degeneration in 7-day-old *norpA;tadr* flies.

D, Relative sizes of peripheral rhabdomeres measured in TB staining assays. The asterisk (*) indicates significant differences between the paired samples.

E, In optical neutralization assays, the initial speed of rhabdomere loss in the *norpA;tadr* fly was the same as that in *tadr* mutant, and was much faster than in the *norpA* single mutant.

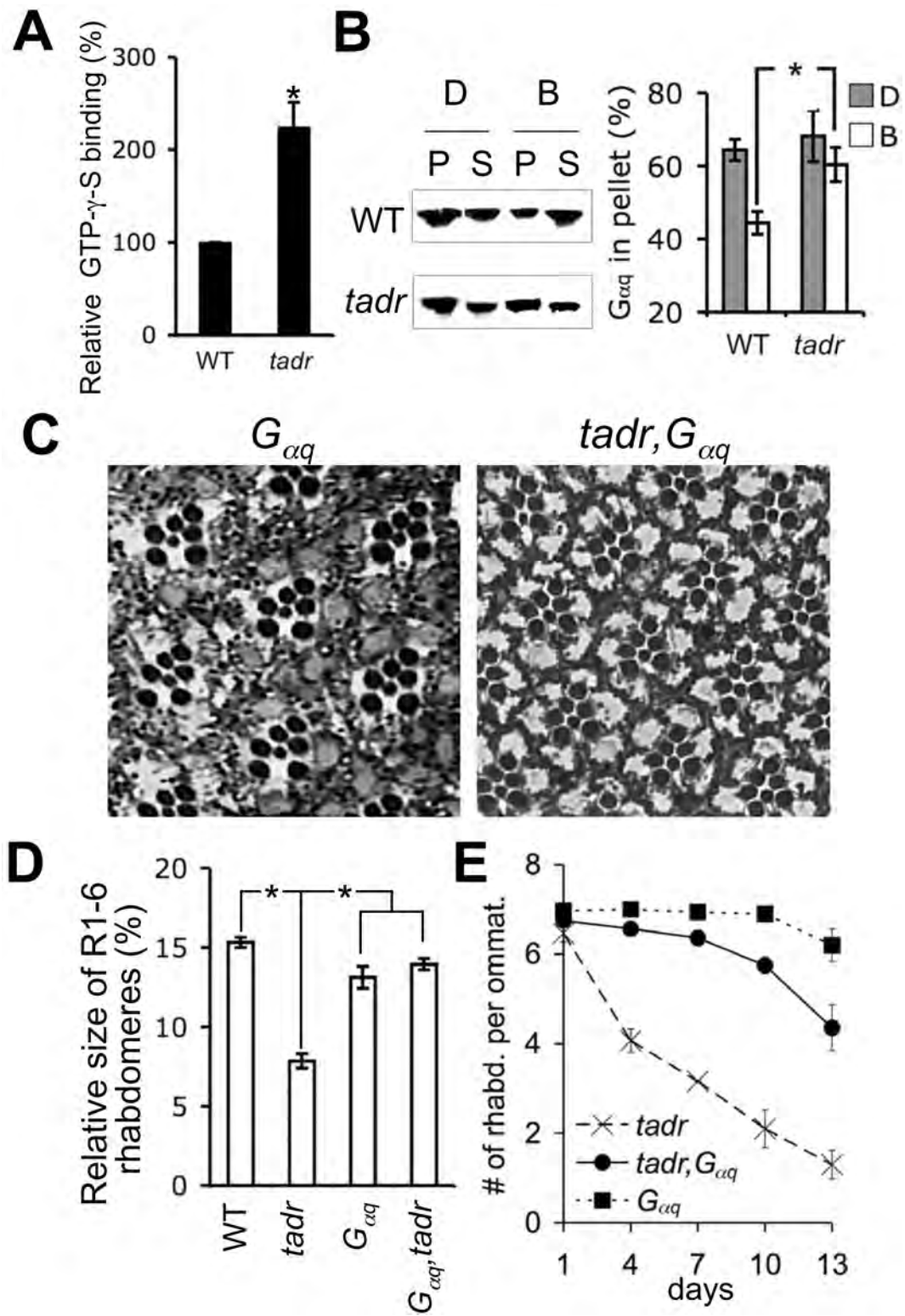


Figure 2.8

Figure 2.8. An abnormal G_q signaling mediates the *tadr* rhabdomeral degeneration.

A, The light-stimulated $GTP\gamma S$ binding (see Materials and Methods for the measurement) in the *tadr* mutant membrane sample was much higher than in wild type. Shown are the relative levels of $GTP\gamma S$ binding with the wild type set as 100%. Data from three experiments were averaged. The asterisk (*) indicates significant differences from the wild-type controls.

B, Blue-light treatment did not efficiently dissociate $G_{\alpha q}$ from membrane in the *tadr* mutant. Heads were collected from dark-reared flies either directly (D) or after 1 h exposure to blue light (B). After homogenization, supernatant (S) and membrane pellet (P) fractions were subjected to Western blot. The percentages of $G_{\alpha q}$ in the membrane pellet were quantified using NIH ImageJ software. The averaged data of four independent experiments is shown in the right panel. The asterisk (*) indicates significant differences from the wild-type controls.

C, Reduction of $G_{\alpha q}$ level suppressed rhabdomeral degeneration in a *tadr;G $_{\alpha q}$* double mutant at 7 days. The $G_{\alpha q}$ allele is $G_{\alpha q}^1$.

D, Relative sizes of peripheral rhabdomeres measured in TB staining assays. The asterisk (*) indicates significant differences between the paired samples.

E, In optical neutralization assays, the number of visible rhabdomeres in *tadr;G $_{\alpha q}$* flies decreased much more slowly than in the *tadr* single mutant.

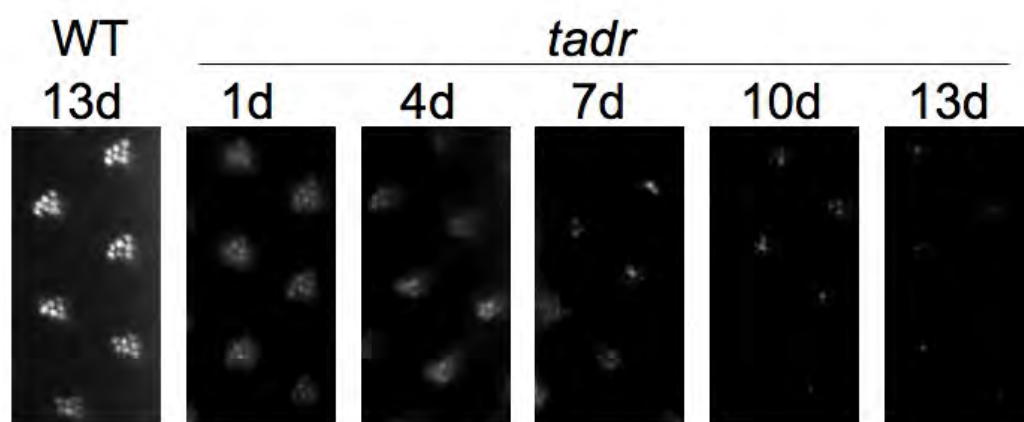


Figure 2.9

Figure 2.9. In optical neutralization assays, the number of visible rhabdomeres decreases gradually during the aging of *tadr* mutant flies.

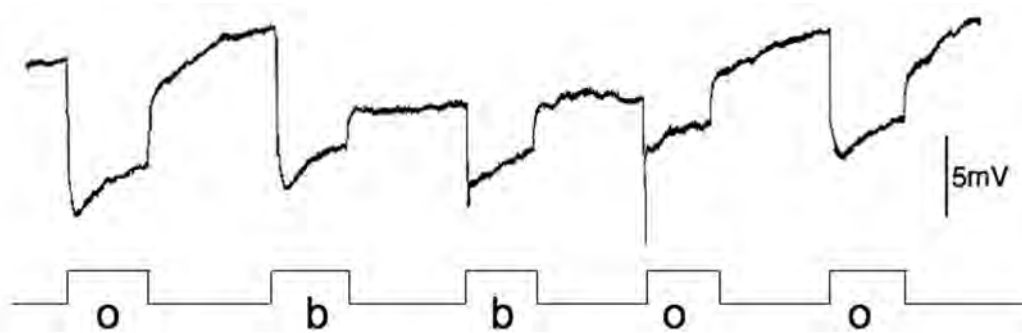


Figure 2.10

Figure 2.10. In ERG recordings, blue light induces prolonged depolarization afterpotential (PDA) in 7-day-old *tadr* mutant. Both orange (o) and blue (b) light pulses are 4 seconds. As PDA is triggered through excessive Rh1 activities specifically in peripheral photoreceptors (Dolph et al., 1993), this data indicates that the peripheral photoreceptors in the examined flies are light responsive.

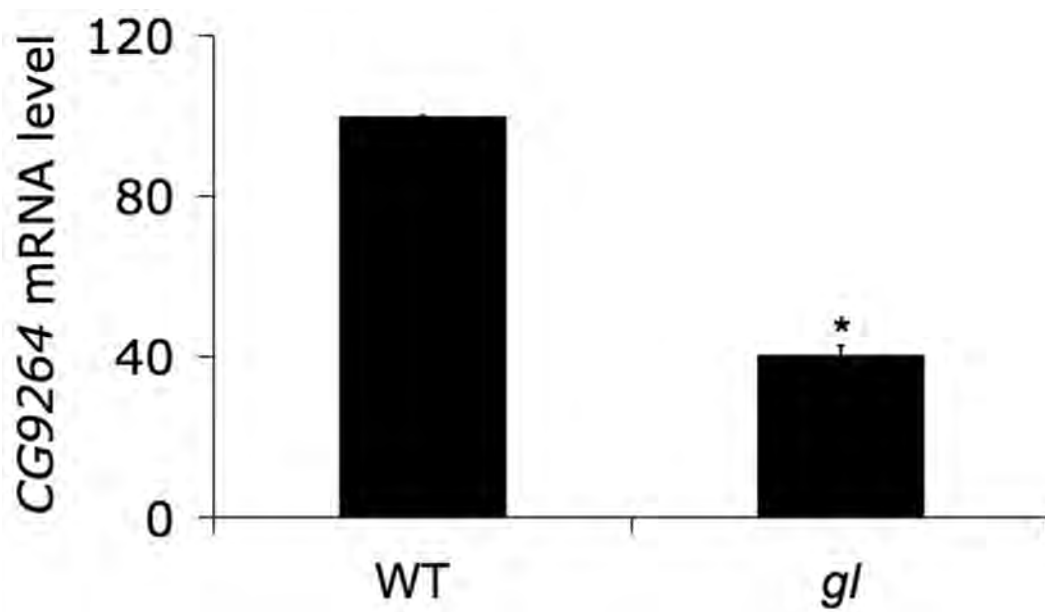


Figure 2.11

Figure 2.11. Real-time RT-PCR data show that *tadr* (*CG9264*) is highly expressed in the photoreceptors. The mutant *glass*² (*gl*) contains no photoreceptor. Total RNAs were extracted from 2-day-old fly heads using Trizol reagent (Invitrogen). Real-time RT-PCR was conducted with an ABI PRISM7700 and a SuperScript III Platinum One-Step kit (Invitrogen). The relative mRNA level was calculated by setting the wild-type *CG9264* level as 100%. A *Rpl32* gene was used as an internal control. Primers for *rpl32*: GCAAGTGTGCGGCTCGTATT/ TGGTGGCGGATGAAGTGCTT (216bp); primers for *CG9264*: ATGGGGTCAGCGTCGGGATC/CCGACTGTCATCTGTGCTCG (131bp).

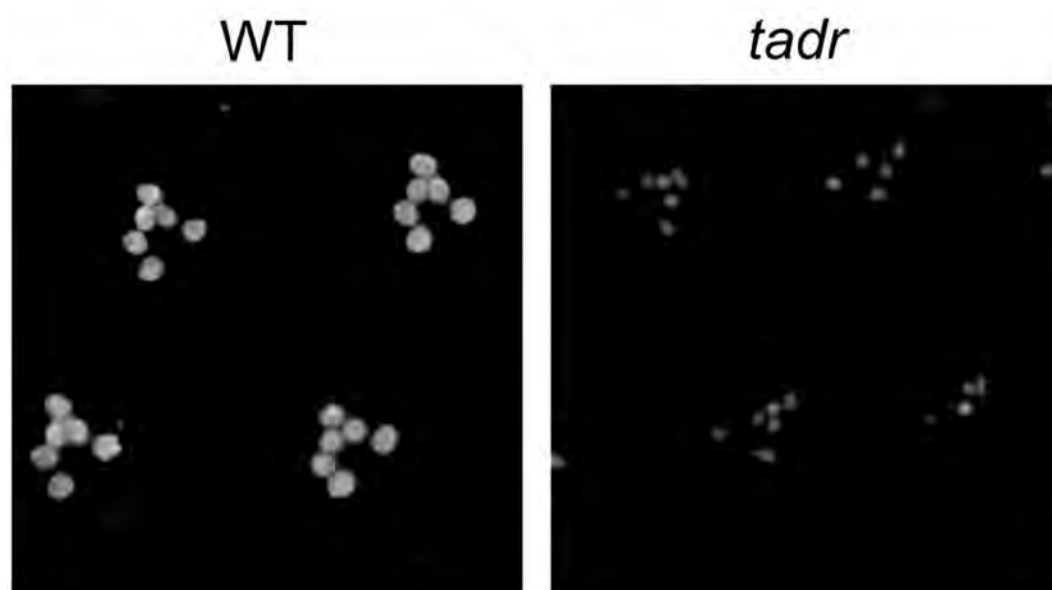


Figure 2.12

Figure 2.12. Immunostaining of eye sections from 10-day-old flies fails to reveal excessive endocytosis of Rh1 in *tadr* mutant photoreceptors. Rh1 protein was localized almost exclusively in the rhabdomere in both wild-type and mutant flies. Flies were raised in a normal 12 hr light /12 hr dark cycle.

**CHAPTER III: A CUB- AND LDLA-DOMAIN
PROTEIN ANTAGONIZES RHODOPSIN
ENDOCYTOSIS TO MAINTAIN *DROSOPHILA* VISUAL
SENSITIVITY**

This work was conducted under the direction of Dr. Hong-Sheng Li. My contribution in this work was to execute the majority of the experiments including generating transgenic flies and double mutants, immunostaining, Western blot, electroretinogram recordings and intracellular recordings, generating the CULD antibody, glutathione-sepharose binding assay and amylose resin binding assay. Keith Reddig and Dr. Junhai Han contributed by conducting electron microscopy. Ping Gong contributed by generating the Arr1 antibody. Dr. Hong-sheng Li and I together wrote the manuscript.

3.1 Abstract

The high sensitivity of the photoreceptor neuron to light is critical for animal vision in dim light conditions. A primary determinant of photoreceptor sensitivity is the density of rhodopsin, a light-stimulated G protein-coupled receptor (GPCR), in photoreceptive membrane. In the case of non-visual GPCRs, continuous receptor activation will trigger arrestin-mediated endocytosis to decrease receptor densities in the membrane, which is an important process for many cells to adapt to environmental changes. It is puzzling how photoreceptors can maintain the membrane density of rhodopsin and their continuous sensitivities during light stimulations, although visual arrestin-mediated endocytosis does occur. Here we report that a *Drosophila* CUB- and LDLa-domain transmembrane protein CULD counteracts arrestin-mediated endocytosis to retain rhodopsin in the membrane of the rhabdomere, the light sensory organelle of the *Drosophila* photoreceptor. CULD is mostly localized in the rhabdomere but is also detected in scarce rhodopsin endocytic vesicles that contain an arrestin Arr1. An intracellular region of CULD interacts with Arr1 *in vitro*. In both *culd* mutant and knockdown flies, a large amount of rhodopsin is mislocalized in the cell body of photoreceptors through light-dependent, Arr1-mediated endocytosis, leading to reduction of photoreceptor sensitivity. Expressing a wild-type CULD protein in photoreceptors, but not a mutant variant lacking the Arr1-interacting site, rescues both the rhodopsin mislocalization and the low sensitivity phenotypes. Once rhodopsin has been internalized in adult mutant flies, it is reversed only by expression of CULD but not by blocking the endocytosis, suggesting that CULD promotes recycling of endocytosed rhodopsin to the rhabdomere. Our results demonstrate an important role of CULD in the maintenance of membrane rhodopsin density and photoreceptor sensitivity. We propose that a common cellular function of CUB- and LDLa-domain proteins, in both mammals

and invertebrates, is to concentrate receptors including GPCRs in particular regions of cell membrane.

3.2 Introduction

High visual sensitivity is a common but important characteristic of animal eyes. It is especially critical for nighttime vision. In animal eyes, photoreceptors are the first to receive the incoming rays of light. To function in dim light, photoreceptors have developed utmost sensitivities. It is reported that mammalian rod photoreceptors are capable of detecting single photons (Rieke and Baylor, 1996; Squire et al., 2003).

The high sensitivities of photoreceptors largely depend on a high content of rhodopsin, the light receptor, in the photosensory membrane. In addition to the tightly packed membrane structure of the light sensory organelle, which extensively increases the sensory membrane area to accommodate a great amount of rhodopsin molecules (Squire et al., 2003; Hardie and Raghu, 2001), the high density of rhodopsin molecules in the photosensory membrane is also critical for the photoreceptor to achieve the utmost sensitivity. It is estimated that rhodopsin molecules occupy about 50% of the membrane surface (Palczewski, 2006).

Rhodopsin is a G protein-coupled receptor (GPCR) and depends on arrestins for deactivation (Alberts et al., 2002; Montell, 1999). In the case of non-visual GPCRs, arrestins not only deactivate the receptors, but also mediate endocytosis of active receptors, thereby causing a reduction of receptor densities on the membrane surface (Claing et al., 2002; Hanyaloglu and von Zastrow, 2008). Thus, to maintain continued high sensitivities, photoreceptors may have developed a mechanism to prevent arrestin-mediated endocytosis from decreasing the membrane rhodopsin density during light stimulation. The molecules that antagonize rhodopsin endocytosis to maintain the photoreceptor sensitivity have yet to be identified.

The *Drosophila* eye is a model system for genetic studies of visual function and GPCR

signaling and regulation. This compound eye is composed of 700-800 units named ommatidia, and each ommatidium has 6 peripheral and 2 central photoreceptors (Montell, 1999). The light sensory organelles in all these photoreceptors are tightly packed microvillar structures named rhabdomeres, each of which contains more than 100 million rhodopsin molecules (Zuker, 1996). This extremely high content of rhodopsin in the rhabdomere plays a key role for the *Drosophila* photoreceptor to achieve the utmost sensitivity.

The rhodopsin in all 6 peripheral photoreceptors is Rh1. Like most non-visual GPCRs, active Rh1 can be endocytosed through dynamin-dependent pathways (Alloway et al., 2000; Kiselev et al., 2000; Satoh and Ready, 2005; Han et al., 2007). In wild-type flies, a visual arrestin, Arr1, mediates light- and dynamin-dependent Rh1 endocytosis, at least at the pupal stage (Satoh and Ready, 2005). Nonetheless, the level of endocytosed Rh1 is very low in adult flies, which does not significantly decrease the photoreceptor sensitivity (Han et al., 2007). The regulatory mechanism of Arr1-mediated endocytosis and the fate of the endocytosed Rh1 molecules have remained unknown.

Here we report that a CUB and LDLa-domain transmembrane protein (CULD) counteracts Arr1-mediated endocytosis to localize Rh1 in rhabdomeres, and thereby to maintain high sensitivities of *Drosophila* photoreceptors. CULD binds Arr1 *in vitro*, which is important for the CULD function. We further show that CULD maintains Rh1 rhabdomeral localization at least partially through recycling the endocytosed Rh1 molecules back to rhabdomeres. Our data suggest that CULD and its similar proteins may be a new group of membrane proteins that bind arrestins and maintain the membrane densities of rhodopsin and other GPCRs.

3.3 Materials and Methods

Flies genetics and light treatments

All examined flies except the *UAS-Shi^{ts};ey-gal4;culd*, *Act5-gal4;UAS-culd^{RNAi}* and *Act5-gal4;UAS-culd^{RNAi},arr2* have a *cn,bw* background for the elimination of compound eye screening pigments. The genotype of the wild type is *cn,bw*. The mutant alleles used in this work are *arr1¹*, *arr2⁵*, *G_{αq}¹*. The *culd* mutant (PBac{RB}CG17352^{e01982}), Df(3L)66C–G28, *Act5-gal4* and *tub-gal4* were obtained from the Bloomington *Drosophila* stock center. The *UAS-culd^{RNAi}* line was from the Vienna *Drosophila* RNAi Center (VDRC). Flies were reared at 21°C in approximately 12h light (~700lux)/12h dark cycles unless mentioned otherwise in the text. For dark rearing, the flies were put in complete darkness from the prepupal stages.

The CG17352 EST clone GH01676 was obtained from the *Drosophila* Genomics Resource Center. After subcloning the CG17352 cDNA into a pCaSpeR–*trp* (Han et al., 2006) or a pCaSpeR–*hs* vector, the plasmids were injected into *w¹¹¹⁸* flies to generate *P[trp–CULD]* and *P[hs–CULD]*. For generation of the *P[trp–CULD*]* transgenic fly, the point mutations (S⁷²⁹A, S⁷³¹A, S⁷³³A, T⁷³⁵A) were created in the EST clone through PCR mutagenesis before it was injected into the pCaSpeR–*trp* vector. To express the protein through a heat-shock promoter, fly vials were immersed in a 37°C water bath for 1 hour, 4 times a day.

Electrophysiological recordings

Electroretinograms (ERGs) were examined as previously described (Ni et al., 2008). In brief, flies were immobilized with thin stripes of lab tape. Two glass microelectrodes filled with Ringer's solution were separately put on the eye surface and the thorax (as reference). The signal was amplified and recorded using a Warner IE210 Intracellular Electrometer.

Intracellular recordings were performed as previously described (Han et al., 2007) with minor modifications. After the fly was immobilized with lab tapes, a small opening was made on surface of the eye using fine tweezers. A thin glass microelectrode with resistance $>30\text{M}\Omega$ (filled with 2M KCl) was gradually inserted into the opening until light-induced depolarization of membrane potential was observed. The reference electrode, filled with Ringer's solution, was put on the surface of the same eye.

In both ERG and intracellular recordings, orange light of different intensities (through passing a series of neutral density filters, each has 20% transmission for orange light) was used to stimulate responses. After dark adaption for 3 minutes, the fly eye was stimulated with a series of 2-second light pulses of increasing intensities.

The relative light sensitivity is defined as I_{WT}/I_M , where I_{WT} represents the mean light intensity required to stimulate a visible response in wild-type flies, and I_M is the lowest light intensity that is necessary for stimulation of a response in the examined mutant fly. For each genotype and condition, ≥ 6 flies were examined and the relative sensitivities were averaged to obtain a mean. The standard errors of means (s.e.m.) were calculated and presented as error bars in figures.

Electron Microscopy (EM)

Fly heads were bisected and fixed in a solution of 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer (pH7.4). After three times of wash, fly heads were post-fixed with 1% osmium tetroxide for 1 hour, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Spurr's resin. Thin sections of eyes were cut, stained with lead citrate and uranyl acetate, and examined using a transmission electron microscope.

To localize Rh1 in photoreceptors at the EM level, fly heads were fixed and embedded in LR White as described (Han et al., 2007). Thin sections of eye were cut and immunostained with a monoclonal Rh1 antibody and anti-mouse IgGs conjugated with 15-nm gold

particles. After staining with 1% aqueous uranyl acetate, the sections were examined under the transmission electron microscope.

Antibodies

The CULD antibodies were raised in rabbits against a GST-fused intracellular fragment (aa 551–738). The Arr1 antibodies were raised against a C-terminal fragment (aa 226–319). The Arr2 antibody was as described (Han et al., 2006). The sources of other antibodies were DSHB (Rh1), Sigma–Aldrich (G_q) and C. Montell (TRP).

Immunostaining

For immunofluorescence staining of Rh1 and TRP, fly heads were fixed with 4% paraformaldehyde in PBS, dehydrated with acetone and embedded in LR White resin as described (Porter and Montell, 1993). 1- μ m sections were cut and stained with primary antibodies and then FITC- or TRITC-conjugated secondary antibodies.

For the staining of CULD, before the dehydration, fly eyes were pre-stained with anti-CULD antibody overnight. This helped to preserve the antigen during the heating process of embedding.

The percentages of Rh1 mislocalization were calculated by the ratio of SIC to SIO, where SIC represents the signal amount in cell body and SIO represents the signal amount in the entire ommatidium. The amount of Rh1 signal was measured with NIH ImageJ software. At least four sets of data were averaged for each genotype and/or light treatment.

Glutathione–sepharose binding assay and amylose resin binding assay

For glutathione–sepharose binding, cDNA fragment encoding the intracellular fragment aa 551–738 was amplified through PCR and inserted into a pGEX–5X vector to express GST-fused protein (GST–C) in bacteria. The fusion protein was immobilized on glutathione–sepharose beads (Amersham). For amylose resin binding, cDNA fragments encoding a series of intracellular fragments were amplified through PCR and inserted into

a pMAL-c2 vector to express MBP-fused proteins in bacteria. The fusion proteins were immobilized on amylose resin (NEB). The proteins on beads/resin were incubated with wild-type fly head extracts in PBS that contains 0.5% Triton X-100 and protease inhibitors (Roche). After three washes with the incubation solution, the bound protein was eluted and subjected to SDS-PAGE and Western blot.

3.4 Results

3.4.1 Photoreceptor sensitivity is reduced in the *culd* mutant

A previous microarray analysis identified 128 genes with high expression levels in the *Drosophila* eye (Xu et al., 2004). From the Bloomington *Drosophila* Stock Center, we obtained 41 P element-insertion flies that are potential mutants of those eye-expressed genes, and examined their responses to light through electroretinogram (ERG) recordings. In one P-insertion fly, stimulation of a visible ERG response required a much higher light intensity compared to that for wild type (Fig 3.1A), at least at an age older than 4 days. In addition, the response of this mutant terminated slow in most 4-day-old flies. As the P element in this mutant is inserted in the 3rd intron of the gene *CG17352*, which encodes a membrane protein containing a CUB and an LDLa domain in the extracellular part, we named the mutant *culd*.

Based on the ERG data, we calculated the relative light sensitivities of 4-day-old flies, as described in the Methods. The result showed that the light sensitivity of the *culd* mutant was approximately 100-fold lower than that of wild type (Fig 3.1B). As ERG records the summed potential change caused by electrical activities of both photoreceptors and secondary neurons (Montell, 1999; Pak and Leung, 2003), to investigate whether the lower sensitivity of ERG response in the *culd* mutant is due to a defect in the photoreceptor, we further recorded intracellular responses of single photoreceptors to light. We examined the light sensitivities of photoreceptors in 4-day-old flies, and found the sensitivity of the *culd* photoreceptor was also reduced by about 100 folds compared to that of wild type (Fig 3.1C and D).

3.4.2 Rh1 rhodopsin is mislocalized to the cell body of the *culd* photoreceptor due to Arr1-dependent endocytosis

To find out why the photoreceptor sensitivity is reduced in the *culd* mutant, we examined the rhabdomere structure and the levels of visual signaling components. Given that degeneration of rhabdomeres always leads to reduction of visual sensitivity, we first investigated the morphology of rhabdomeres in the mutant by electron microscopy (EM). The EM pictures did not reveal any obvious difference in the rhabdomere between wild type and the mutant (Fig 3.2A). Next, using Western blot we examined the protein levels of known visual signaling molecules, including Rh1, G_q, PLC and TRP, in the mutant, and found they were all comparable to wild type (Fig 3.2B). However, in immunostaining experiments we detected an abnormality in the subcellular distribution of Rh1, the rhodopsin of all peripheral photoreceptors.

In cross eye sections of the *culd* mutant, the light-stimulated channel TRP was localized normally in rhabdomeres (Fig 3.2C). In contrast, about 30% of Rh1 was internalized to the cell body of the mutant photoreceptor at the age of 2 days (Fig 3.2C, D and E). When the fly became 4 days old, the proportion of mislocalized Rh1 increased to about 60% (Fig 3.2D and E). By immunogold labeling of Rh1 at the EM level, we found that the mislocalized Rh1 appeared to reside in a great amount of tiny vesicles that form multivesicular body-like structures in the *culd* photoreceptor (Fig 3.2F).

Interestingly, in dark-reared *culd* flies that had never been exposed to light from the early pupal stage, Rh1 was localized normally in peripheral rhabdomeres (Fig 3.3A and E). This may suggest that the Rh1 mislocalization occurs through activity-dependent endocytosis. As most GPCRs including Rh1 are endocytosed in dynamin-dependent manners (Ferguson, 2001), we introduced a temperature-sensitive, dominant-negative form of the *Drosophila* dynamin Shibire, Shi^{ts}, into the *culd* mutant to test its effect on the Rh1

mislocalization. The result showed that suppression of Shibire function at a restrictive temperature (31°C) virtually abolished the Rh1 mislocalization (Fig 3.3B). Together, these observations suggest that Rh1 is internalized through activity- and dynamin-dependent endocytosis.

Three rhodopsin-interacting molecules, G_q and two visual arrestins Arr1 and Arr2, are known to mediate activity- and dynamin-dependent Rh1 endocytosis (Alloway et al., 2000; Kiselev et al., 2000; Satoh and Ready, 2005; Han et al., 2007). To find out which pathway leads to the Rh1 mislocalization in the *culd* mutant, we separately introduced $G_{\alpha q}^1$ and *arr1*¹, the hypomorphic mutations of the $G_{\alpha q}$ subunit and the Arr1 genes, respectively, into the *culd* mutant, and found that *arr1*¹, but not $G_{\alpha q}^1$, greatly inhibited the mislocalization of Rh1 in the *culd* mutant background (Fig 3.3C, D and E).

As the *arr2* gene is close to the *culd* gene on the chromosome, it is very difficult to introduce an Arr2 mutation into the *culd* mutant background through chromosomal recombination. Thus we used a different strategy to test the role of Arr2 in the Rh1 mislocalization phenotype in the *culd* mutant. We knocked down the CULD expression level using a *culd*^{RNAi} line from the VDRC stock center, and found that the knockdown of CULD expression caused an even higher level of Rh1 mislocalization in an *arr2* null mutant background compared to that in a wild-type background (Fig 3.8). This difference could be due to more Arr1 molecules binding to Rh1 in the absence of competition from Arr2. These data suggested that the mislocalized Rh1 in the *culd* mutant photoreceptor is endocytosed through Arr1 but not Arr2 or G_q .

3.4.3 The mislocalization of Rh1 causes the sensitivity reduction in the *culd* mutant

As the overall level of Rh1 in the *culd* mutant was the same as that in wild type (Fig 3.2B), the mislocalization of Rh1 should result in a decrease of Rh1 concentration in the

rhabdomere, thereby causing the reduction of light sensitivity in the *culd* photoreceptor. This hypothesis is strongly supported by the following observations. First, the Rh1 mislocalization and the sensitivity reduction (Fig 3.2E and Fig 3.1B, respectively) in the *culd* mutant were temporally correlated with each other. Second, the light sensitivity of dark-reared *culd* flies, in which Rh1 is localized normally in rhabdomeres, was the same as that of wild type (Fig 3.4A). Third, the light sensitivity was significantly increased in the *culd* background by blocking dynamin-dependent endocytosis (Fig 3.4B). Finally, in the *arr1;culd* double mutant that has virtually normal Rh1 localization, the light sensitivity was the same as that in the *arr1* single mutant (Fig 3.4C).

3.4.4 Loss of CULD is responsible for the *culd* mutant phenotypes

Given that the P element in the *culd* mutant is inserted within the gene and that the RNAi knockdown of *culd* gene phenocopies the *culd* mutant (Fig 3.8 and Fig 3.9), it is very likely that the mutant phenotypes are due to loss of CULD protein. To confirm this, firstly, we generated a polyclonal antibody against an intracellular fragment of CULD, aa 551-738 (Fig 3.5A), to demonstrate the loss of CULD in the mutant. The antibody revealed strong CULD signals in rhabdomeres of wild-type flies in immunostaining assays (Fig 3.5B). In contrast, no CULD signal was detected in eye sections of the *culd* mutant. Secondly, we generated a heterozygous fly with the *culd* mutation over a deficiency chromosome (Df(3L)66C-G28) that lacks the *culd* gene. This fly displayed the same phenotypes as the *culd* homozygous fly (Fig 3.5C, F and G). Thirdly, we excised the P element out of the mutant and found that both the Rh1 localization and the light sensitivity were restored to wild type (Fig 3.5D, F and G). Most importantly, expression of a wild-type *culd* cDNA specifically in the photoreceptor, through a *trp* promoter, rescued both the Rh1 localization and the sensitivity phenotypes of the *culd* mutant (Fig 3.5E, F and G). Thus, loss of CULD

is indeed responsible for the *culd* mutant phenotypes.

3.4.5 CULD interacts with Arr1 to localize Rh1 in the rhabdomere

CULD is a single transmembrane protein consisting of 965 amino acid residues. It has an N-terminal signal peptide, a CUB domain and an LDLa domain in the extracellular region, but has no conserved domain in the intracellular part (Fig 3.5A). How does this protein counteract Arr1-mediated endocytosis to localize Rh1 in the rhabdomere? To address this, we tested whether an intracellular fragment of CULD interacts with Rh1 or Rh1-associated molecules. We fused the CULD fragment aa 551-738 to a glutathione-S-transferase (GST) protein, immobilized it to glutathione-sepharose beads, and used the beads to pull down proteins from head extracts of wild-type flies. The result indicated that the CULD fragment specifically pulled down Arr1, but not Rh1, Arr2 or G_q (Fig 3.6A).

Since the CULD antibody did not appear to work for immunoprecipitation, we could not use co-immunoprecipitation assays to confirm the CULD-Arr1 interaction *in vivo*. Thus, we decided to directly test the functional significance of the interaction using transgenic flies. For this purpose, we further mapped the Arr1-interacting site of CULD protein with smaller CULD fragments that are fused to a maltose binding protein (MBP). In amylose resin binding assays, a small fragment, aa 717-738, pulled down Arr1 (Fig 3.6B). We created four point mutations in this region, S⁷²⁹A, S⁷³¹A, S⁷³³A and T⁷³⁵A, and found they together abolished the interaction between CULD and Arr1 (Fig 3.6B).

Next we generated a transgenic fly *P[trp-CULD*]* that expresses a mutant CULD protein containing those four mutations. In the *culd* mutant background, expression of this mutant CULD did not fully suppress the Rh1 mislocalization phenotype (Fig 3.6C). In contrast, when a wild-type CULD protein was expressed in the mutant background, the Rh1 localization was not significantly different from wild-type flies. Thus, the interaction

with Arr1 is important for the CULD function *in vivo*.

3.4.6 CULD promotes Rh1 recycling

CULD may interact with Arr1 to control the Rh1 localization in different ways. First, it may prevent Arr1 from inducing Rh1 endocytosis at the very beginning; Second, it may promote the recycling of endocytosed Rh1 back to the rhabdomere, for example, by dissociating Arr1 from Rh1; Third, it could sort endocytosed Rh1 protein to lysosome for degradation. However, the last possibility is not likely because of the following reasons: 1) If Rh1 degradation is impaired in the *culd* mutant, it may cause the accumulation of endocytosed Rh1 but should not lead to the reduction of photoreceptor sensitivity. The sensitivity reduction suggests a decrease of Rh1 concentration in the rhabdomere. 2) In the *culd* mutant we did not detect a significant increase of the overall Rh1 level, which should be expected if the degradation of endocytosed Rh1 is blocked. Thus, CULD may either negatively control Arr1-mediated Rh1 endocytosis, or promote the recycling of endocytosed Rh1 molecules.

In light exposed wild-type flies, we found that CULD protein colocalized with Rh1 in scarce endocytic particles (Fig 3.7A). This led us to suspect that CULD may have a role in the recycling of endocytosed Rh1. To investigate this, we decided to test whether expression of CULD at a later time could remove the previously endocytosed Rh1 from the cell body part and restore the sensitivity of the photoreceptor. For this purpose, we generated a transgenic fly *P[hs-CULD];;culd*, which expresses a wild-type *culd* cDNA in the *culd* mutant background under the control of a heat-shock promoter. We raised the flies in normal light/dark cycles to about 36 hours old to induce Rh1 mislocalization, and then divided them into two groups: the control group was saved in the dark for 2 days without heat shock, while the experimental group was put in the dark for the same

time and was treated with 1-hour heat shock every 6 hours to trigger the expression of CULD protein. Suppression of Rh1 endocytosis by light deprivation did not reverse the Rh1 mislocalization in the control group (Fig 3.7B and C), indicating that the previously mislocalized Rh1 cannot be removed simply upon suppression of endocytosis. However, the heat shock-triggered CULD expression almost fully reversed the Rh1 mislocalization (Fig 3.1B and C). More importantly, the light sensitivity also returned to the wild-type level in the heat shocked *P[hs-CULD];;culd* flies (Fig 3.7D), suggesting a recovery of Rh1 concentration in the rhabdomere. In control experiments, heat shock did not cause any significant change in either the Rh1 localization or the light sensitivity of wild type and *culd* mutant flies (Fig 3.7B, C and D). Taken together, these observations strongly suggest that CULD promotes the recycling of endocytosed Rh1 molecules.

3.5 Discussion

In this study, we have identified a *Drosophila* CUB and LDLa domain protein CULD, which binds the visual arrestin Arr1 and antagonizes Rh1 endocytosis to maintain the photoreceptor sensitivity, at least partially through promoting Rh1 post-endocytic recycling. This is the first demonstration that the light receptor rhodopsin undergoes recycling after being internalized and has solved a long-standing puzzle of how photoreceptors counteract arrestin-mediated endocytosis to maintain continued high visual sensitivity during light stimulation.

Endocytic trafficking pathways of Rh1

Like non-visual GPCRs, *Drosophila* Rh1 rhodopsin undergoes activity/light-dependent endocytosis through dynamin-mediated pathways (Alloway et al., 2000; Kiselev et al., 2000; Satoh et al., 2005; Han et al., 2007). In addition to Arr1, another visual arrestin Arr2 and the G_q protein also mediate light-dependent Rh1 endocytosis, at least in mutant flies. In both Arr2 and G_q pathways, the endocytosed Rh1 molecules are sorted to degradation (Orem and Dolph, 2002; Han et al., 2007), probably in lysosomes. However, the fate of Rh1 molecules that are endocytosed through the Arr1 pathway remained unknown. In this work, we have found that the Arr1-endocytosed Rh1 protein is not degraded in the *culd* mutant, and the CULD protein is involved in the recycling of endocytosed Rh1. Together, these observations suggest that the Rh1 molecules in the Arr1 endocytic pathway are directed to recycling.

The Arr2-dependent Rh1 endocytosis leads to apoptotic cell death, which is only observed in mutant flies (Alloway et al., 2000; Kiselev et al., 2000). The G_q-dependent endocytic activity is not obvious in wild-type flies either, because of the rapid deactivation of Rh1 by Arr2 (Dolph et al., 1993) and a dCAMTA/dFbx14 mechanism (Han et al., 2007).

It is reported that Arr1 mediates light-dependent Rh1 endocytosis in wild-type flies at the pupal stage (Sato et al., 2005). However, the level of endocytosed Rh1 is very low in adult flies. Here we show this is due to the counteraction of the CULD protein.

It remains a mystery why Rh1 has to be endocytosed by Arr1 and then recycled back. A likely explanation is that some photo-converted Rh1 molecules may need a longer time to change back to the inactive, functional form, and this process has to take place outside the rhabdomere to prevent abnormal Rh1 activities from damaging the membrane structure of the rhabdomere. Indeed, it has been found that *arr1* mutants undergo rhabdomeral degeneration (Sato et al., 2005).

Rhodopsin internalization and photoreceptor sensitivity

In our study, the level of sensitivity reduction in the *culd* mutant is not strictly proportional to the percentage of internalized Rh1. This could be due to the fact that we have underestimated the level of internalized Rh1, because some endocytosed Rh1 molecules may still reside within the microvilli of the rhabdomere. Alternatively, the decrease of Rh1 concentration in the rhabdomere may not be the only reason of the sensitivity reduction. The internalization of a large amount of Rh1 may have changed the lipid composition of the rhabdomeral membrane, which has a great impact on the visual signaling. It has been reported that loss of docosahexaenoic acid in mammalian outer segments causes a lower level of rhodopsin activity and reduced visual signaling efficiency (Niu et al., 2004; Barnett-Norris et al., 2005).

In addition to the sensitivity reduction, the light responses terminate slowly in the *culd* mutant. This phenotype also depends on the Rh1 internalization (Ni et al., unpublished observations). It could be explained by the potential lipid change in the rhabdomeral membrane, or could be due to that Arr2, the key factor for the Rh1 deactivation and the light response termination, is also mislocalized with Rh1 (Ni et al., unpublished observation).

Mechanisms of CULD function

A variety of GPCRs undergo rapid recycling to the membrane after being endocytosed, which is important for timely recovery of cellular responsiveness to environmental stimuli (Hanyaloglu and von Zastrow, 2008). Although it has been reported that the recycling of a receptor depends on its C-terminal sequence and a couple of binding partners (Hanyaloglu and von Zastrow, 2008; Trejo and Coughlin, 1999; Moore et al., 2007; Vargas and Zastrow, 2004; Kishi et al., 2001; Cong et al., 2001; Cao et al., 1999), the mechanism of recycling is largely unknown. Here we have identified CULD as a molecule that promotes *in vivo* recycling of the *Drosophila* visual GPCR Rh1. Further studies on this CULD-mediated Rh1 recycling pathway may help to reveal general mechanisms that underlie GPCR recycling.

Given that both CULD and Arr1 colocalize with Rh1 in endocytic vesicles (Satoh et al., 2005) and that CULD physically interacts with Arr1, we propose that the CULD-Arr1 interaction mediates the recycling function of CULD. CULD may compete for Arr1 binding with the regenerated Rh1 molecules on endocytic vesicles, so that the free Rh1 molecules can recruit additional recycling factors. An alternative mechanism is that, after being co-endocytosed with Rh1, CULD may form a complex with Arr1 to recruit recycling factors and promote Rh1 recycling. Additional studies on the CULD and Rh1 binding sites of Arr1 may help to differentiate these two possibilities.

Although we have demonstrated the recycling function of CULD, our data do not exclude the possibility that CULD directly controls Rh1 endocytosis. CULD could bind to Arr1 in the rhabdomere and prevent it from inducing Rh1 endocytosis. The accumulation of endocytosed Rh1 in the cell body of the *culd* mutant photoreceptor could be due to both an excessive activity of endocytosis and a failure of recycling. To test whether CULD directly controls the endocytosis, we would first block the recycling of Rh1—for example, by genetically disrupting the function of Rab4/Rab11.

Functions of CUB- and LDLa-domain proteins

Both CUB and LDLa domains mediate protein-protein interaction in numerous functionally unrelated proteins (Bork and Beckmann, 1993; Christensen and Birn, 2002). A large group of membrane proteins contain both CUB and LDLa domains in their extracellular domains, including at least 12 in human and more than 11 in *Drosophila*. So far only a limited number of CUB-LDLa proteins have been functionally studied. For instance, a *C. elegans* protein LEV-10 is found to aggregate postsynaptic ionotropic acetylcholine receptors at neuromuscular junctions (Gally et al., 2004); two human CUB-LDLa proteins NETO-1 and NETO-2, which are predominantly expressed in retina and brain, respectively (Stöhr et al., 2002; Michishita et al., 2003), are reported to be auxiliary proteins of glutamate receptors, and the NETO-1 is critical for the concentration of NR2R-containing NMDA receptors in the postsynaptic density (Ng et al., 2009; Zhang et al., 2009). However, the role of CUB-LDLa proteins in the regulation of GPCR function has not been studied previously.

Our study now demonstrates that the *Drosophila* CUB-LDLa protein CULD is required to localize the visual GPCR Rh1 in the rhabdomeral membrane. Thus we propose that a common cellular function of CUB-LDLa proteins in both mammals and invertebrates is to concentrate membrane receptors including GPCRs in particular regions of cell surface.

3.6 Acknowledgements

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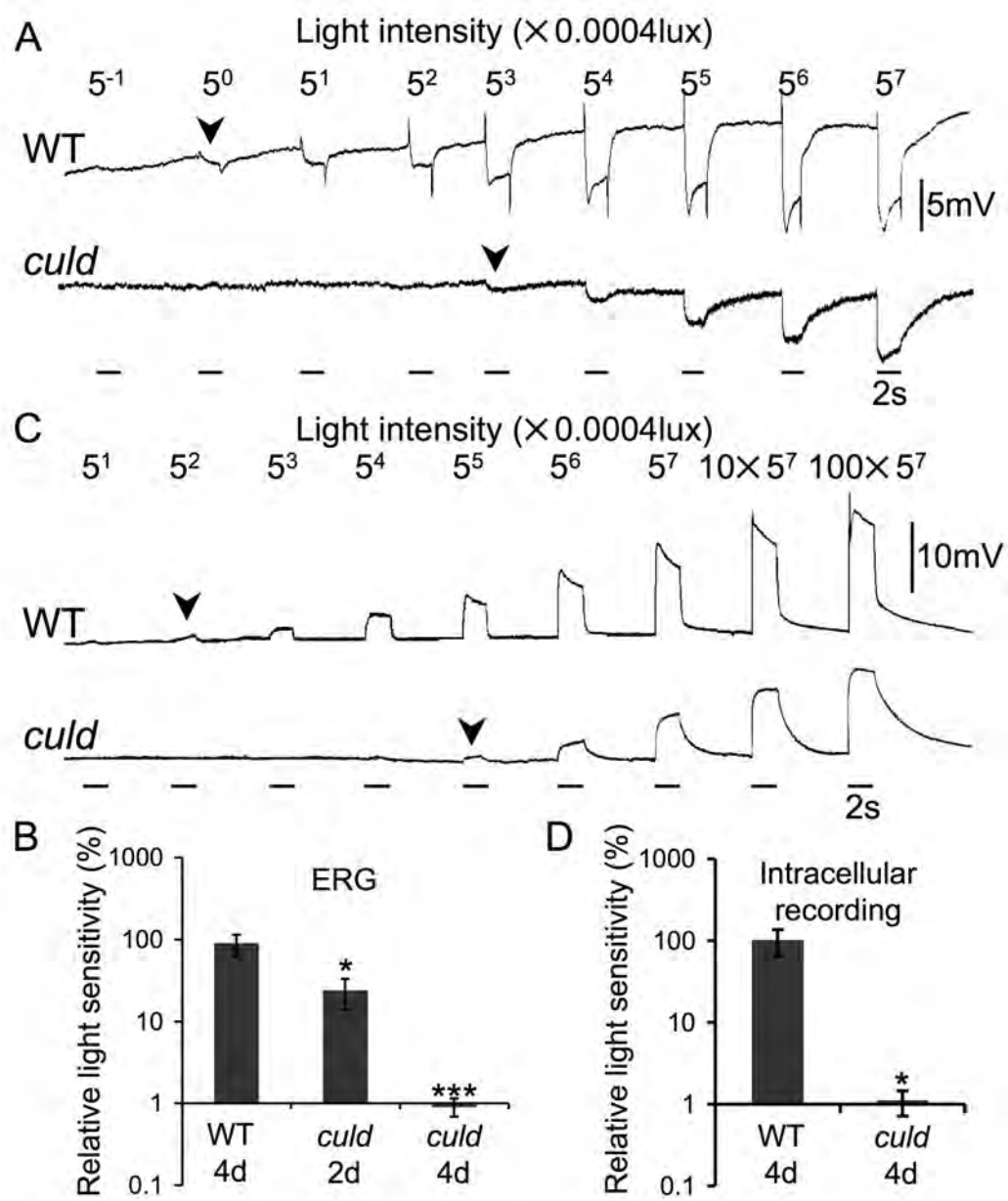


Figure 3.1

Figure 3.1. Photoreceptor sensitivity is reduced in the *culd* mutant.

A. Electroretinogram (ERG) recordings revealed decreased visual sensitivity in 4-day-old *culd* mutant flies. Fly eyes were stimulated with a series of 2-second light pulses of increasing intensities as labeled on the top. The first appearing response is marked with an arrowhead. WT: wild type.

B. Quantification of ERG sensitivities in wild-type and *culd* mutant flies at the marked ages. 2d: 2 days old; 4d: 4 days old. The shown mean relative sensitivities were calculated as described in Materials and Methods. The error bars represent s.e.m.. The asterisk (*) indicates a significant difference from the wild type (*: $P < 0.05$; ***: $P < 0.001$).

C. *in vivo* intracellular recordings of individual photoreceptors revealed decreased photoreceptor sensitivity in 4-day-old *culd* mutant flies. Flies were stimulated with a similar set of light pulses as for ERG.

D. Quantification of photoreceptor sensitivities measured through intracellular recordings in 4-day-old wild-type and *culd* flies. The shown mean relative sensitivities were calculated as for ERG sensitivities. The error bars represent s.e.m.. The asterisk (*) indicates a significant difference from the wild type ($P < 0.05$).

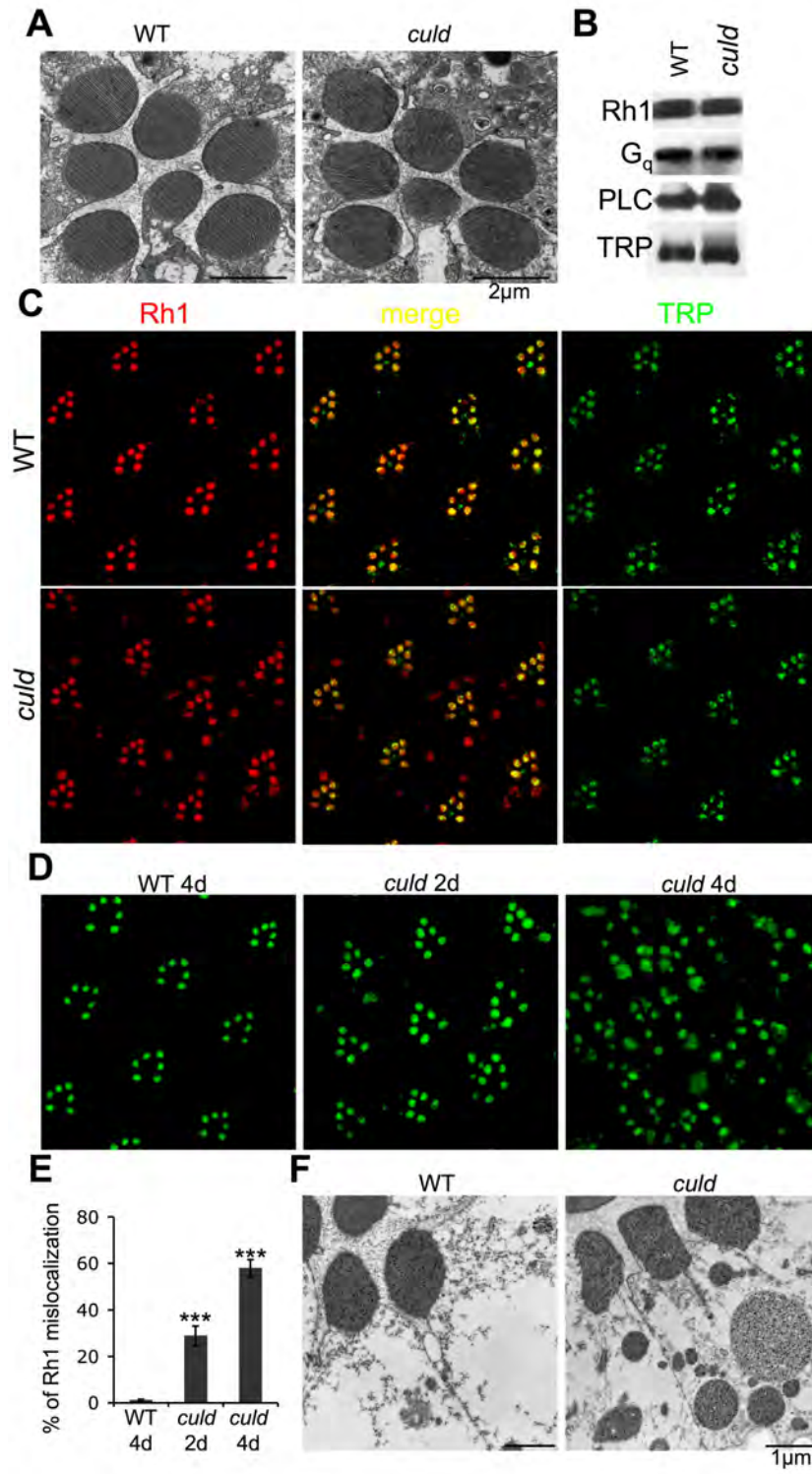


Figure 3.2

Figure 3.2. Rh1 rhodopsin is mislocalized in *culd* mutant photoreceptors.

A. Electron microscopy revealed normal morphology of rhabdomeres in 4-day-old *culd* mutant flies. Each panel shows a single ommatidium in eye cross sections.

B. The protein levels of signaling molecules in the phototransduction cascade did not decrease in the *culd* mutant.

C. In immunofluorescence staining, TRP was localized normally in rhabdomeres, while Rh1 was mislocalized to the cell body regions of *culd* mutant photoreceptors. Eye cross sections of 2-day-old flies were stained.

D. Immunofluorescence staining revealed an even more severe mislocalization of Rh1 in 4-day-old *culd* mutant flies.

E. Quantification of the Rh1 mislocalization in wild-type and *culd* mutant flies at the marked ages. The shown % of Rh1 mislocalization was calculated as described in Materials and Methods. The error bars represent s.e.m.. The asterisk (*) indicates a significant difference from the wild type (***: $P < 0.001$).

F. Immunogold labeling of Rh1 showed that the mislocalized Rh1 in *culd* mutant photoreceptors resided in large collections of tiny vesicles.

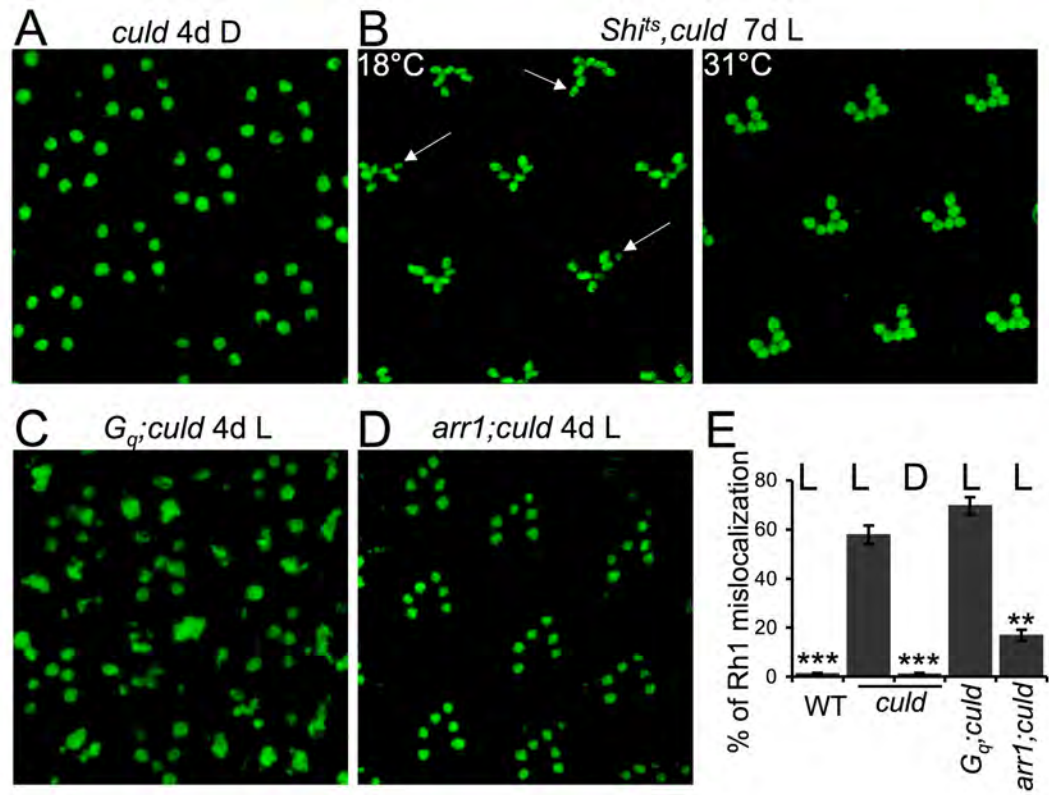


Figure 3.3

Figure 3.3. The mislocalization of Rh1 is due to activity- and Arr1-dependent endocytosis.

A. Rh1 was localized normally in the rhabdomeres of 4-day-old, dark-reared *culd* mutant flies.

B. Blocking of dynamin function through Shi^{ts} , a temperature-sensitive, dominant-negative form of Shibire, prevented the Rh1 mislocalization in *culd* mutant photoreceptors. Rh1 mislocalization (arrow) was observed at 18°C but not at 31°C, which is a restrictive temperature of Shi^{ts} . Because Shi^{ts} has partial dominant effects even at permissive temperatures (Gonzalez-Bellido et al., 2009), the Rh1 mislocalization in the $Shi^{ts};culd$ fly was not as severe as that in the *culd* fly. The less Rh1 mislocalization was also due to the fact that this genotype ($UAS-Shi^{ts};ey-gal4;culd$) contained 4 copies of w^+ and was raised in about 12h (~100lux) light /12h dark cycles.

C. A hypomorphic mutation of $G_{\alpha q}$ (G_q) did not prevent the mislocalization of Rh1 in *culd* mutant photoreceptors.

D. A hypomorphic mutation of *arr1* suppressed the mislocalization of Rh1 in *culd* mutant photoreceptors.

E. Quantification of the Rh1 mislocalization in 4-day-old flies. L: raised in normal light/dark cycles; D: dark-reared. The asterisk (*) indicates a significant difference from the *culd* mutant raised in normal light/dark cycles (**: $P<0.01$; ***: $P<0.001$).

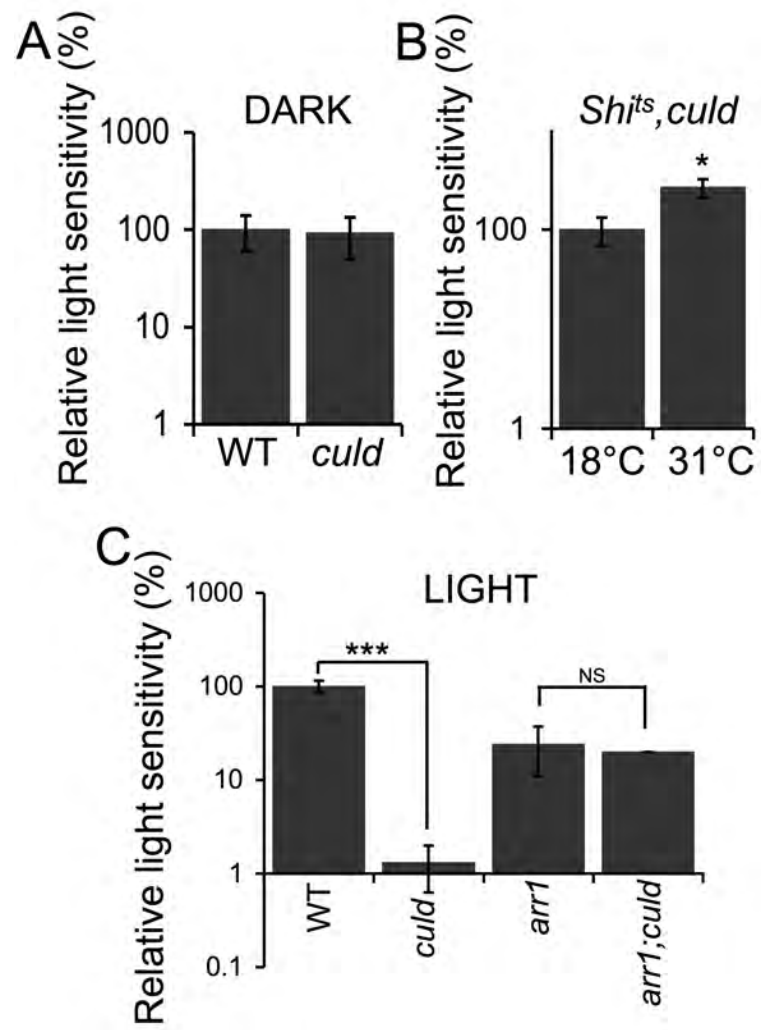


Figure 3.4

Figure 3.4. The mislocalization of Rh1 causes the sensitivity reduction in the *culd* mutant. All sensitivities in this figure are measured by ERG recordings.

A. The visual sensitivity did not decrease in dark-reared, 4-day-old *culd* mutant flies. Note that these flies had no Rh1 mislocalization.

B. Blocking of Rh1 endocytosis through Shi^{ts} suppressed the reduction of visual sensitivity in the *culd* mutant background. The asterisk (*) indicates a significant difference from the flies raised at 18°C (P<0.05).

C. In the hypomorphic *arr1* mutant background that prevents Rh1 mislocalization, the *culd* mutation did not cause a reduction of light sensitivity. The asterisk (*) indicates a significant difference between the paired samples (***: P<0.001). NS: no significant difference.

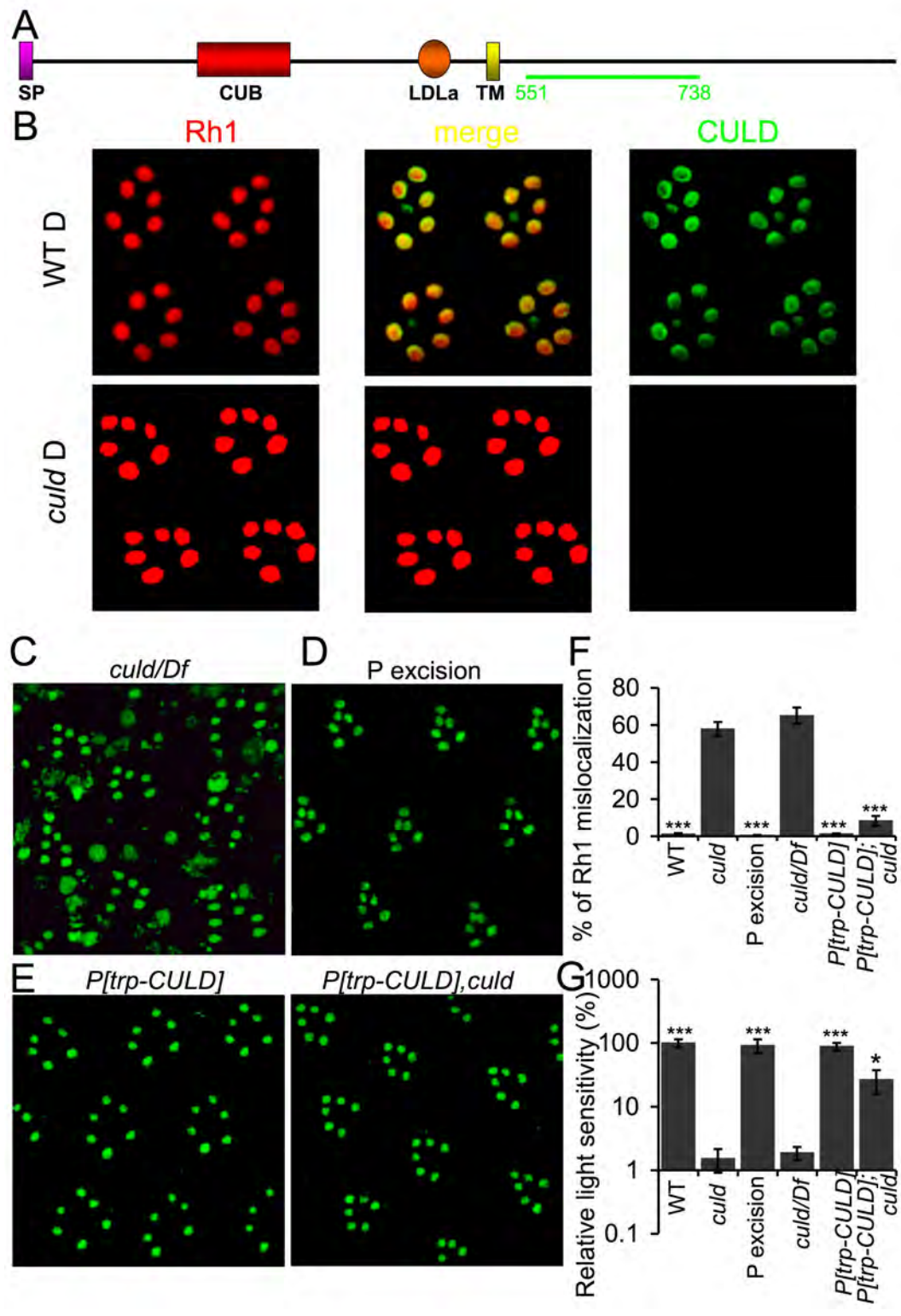


Figure 3.5

Figure 3.5. Loss of CULD is responsible for the *culd* mutant phenotypes.

A. The domain structure of the CULD protein. SP: signal peptide; CUB: Complement C1r/C1s, Uegf, Bone morphogenic protein 1; LDLa: low-density lipoprotein receptor domain class A; TM: transmembrane domain. The fragment aa 551-738 (in green) was used to generate the polyclonal CULD antibody.

B. CULD protein was detected in wild-type but not *culd* mutant flies. In wild type, it was co-localized with Rh1 in peripheral rhabdomeres. Flies were raised in the dark and examined at 2 days old.

C. The heterozygote of the *culd* mutation over the deficiency chromosome Df(3L)66C-G28 showed similar Rh1 mislocalization as the homozygous mutant.

D. A precise excision of the P element from the *culd* mutant rescued the Rh1 mislocalization.

E. A wild-type *culd* cDNA abolished the Rh1 mislocalization (right panel) after being expressed specifically in photoreceptors through a *trp* gene promoter. The left panel is in a wild-type background, shown as a control.

F. Quantification of the Rh1 mislocalization in 4-day-old flies. The asterisk (*) indicates a significant difference from the *culd* mutant (***: $P < 0.001$).

G. Loss of CULD is responsible for the visual sensitivity reduction in the *culd* mutant. The asterisk (*) indicates a significant difference from the *culd* mutant (*: $P < 0.05$; ***: $P < 0.001$).

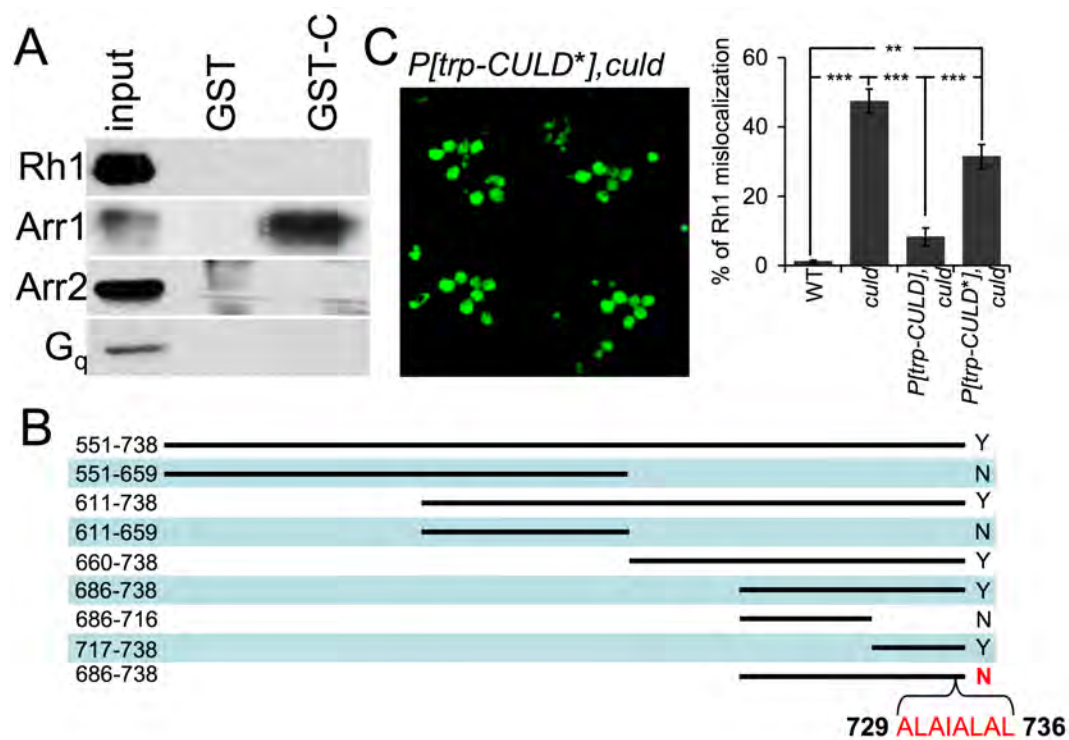


Figure 3.6

Figure 3.6. CULD interacts with Arr1 to localize Rh1 in the rhabdomere.

A. Arr1 interacted with CULD *in vitro*. A GST-fused intracellular fragment of CULD (aa 551-738) specifically pulled down Arr1 from fly head extracts in a glutathione-sepharose binding assay. Lane one was loaded with 1/15 of extract input.

B. Sequential truncation of a MBP-CULD fragment identified four amino acids (S⁷²⁹, S⁷³¹, S⁷³³, T⁷³⁵) critical for the interaction with Arr1. Y and N: the fragment bound or did not bind Arr1, respectively. Note that the fragment aa 686-738 containing the mutations S⁷²⁹A, S⁷³¹A, S⁷³³A, T⁷³⁵A (showed in red) did not bind Arr1.

C. A mutant *culd* cDNA (*CULD**) containing the S⁷²⁹A, S⁷³¹A, S⁷³³A, T⁷³⁵A mutations did not fully suppress the Rh1 mislocalization after being expressed in *culd* mutant photoreceptors through the *trp* gene promoter. The right panel is the quantification of Rh1 mislocalization in 4-day-old flies. The asterisk (*) indicates a significant difference between the paired samples (**: P<0.01; ***: P<0.001).

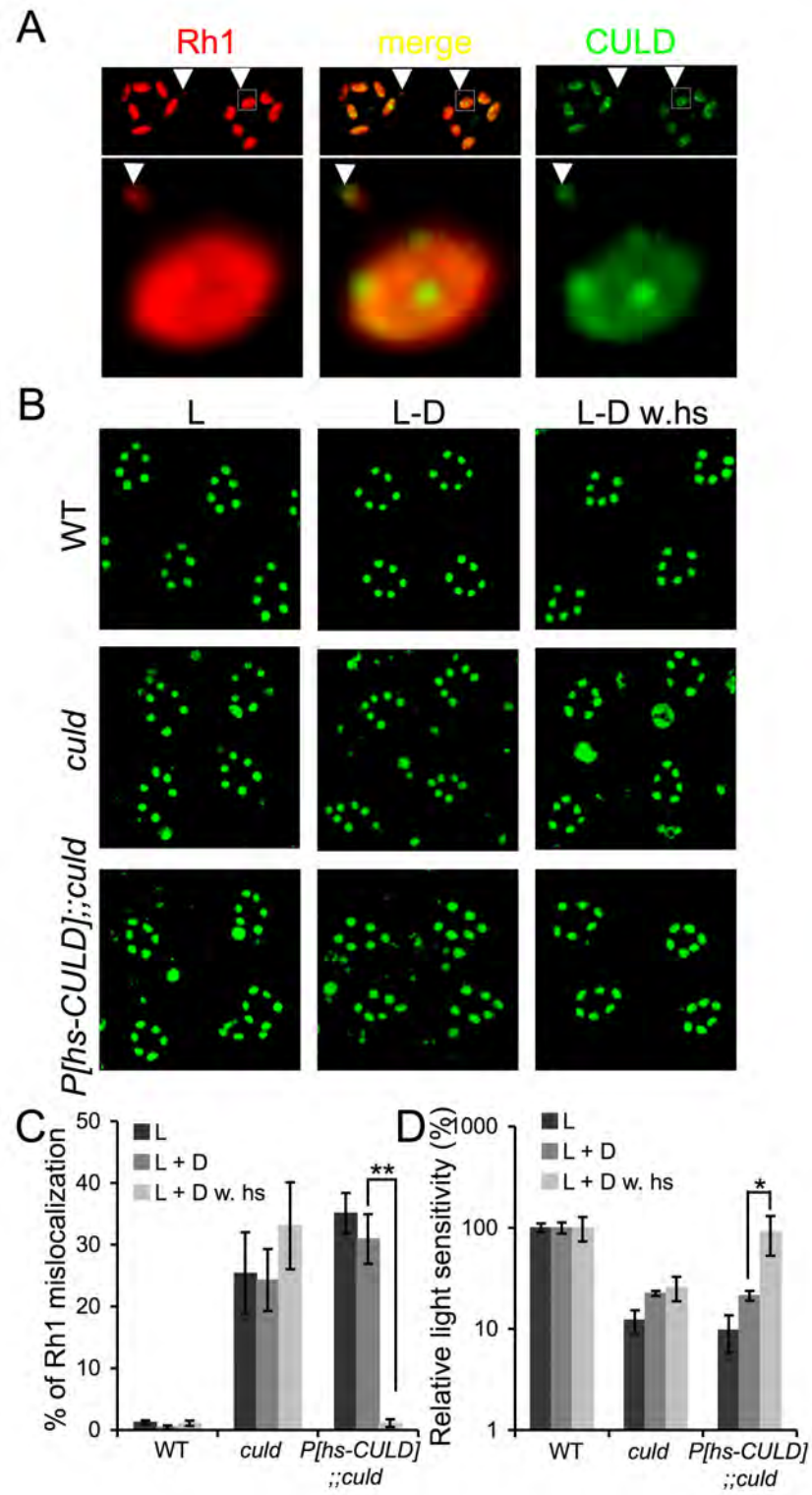


Figure 3.7

Figure 3.7. CULD promotes Rh1 recycling.

A. Upon light stimulation, CULD proteins were internalized and co-localized with endocytosed Rh1 (arrowheads). Flies were raised in normal light/dark cycles and were examined at 2 days old.

B. The previously endocytosed Rh1 was removed by expression of CULD through a heat-shock promoter, but not by suppression of endocytosis through light deprivation. L: raised in normal light/dark cycles for ~36 hours; L+D: after being raised in normal light/dark cycles for ~36 hours, flies were moved to the dark for ~2 days; L+D w. hs: same as L+D except that the flies were treated with 1-hour heat shocks every 6 hours in the dark.

C. Quantification of the Rh1 mislocalization in B (**: $P < 0.01$).

D. The reduction of light sensitivity was reversed by expression of CULD through the heat-shock promoter, but not by light deprivation (*: $P < 0.05$). The treatments were the same as in B.

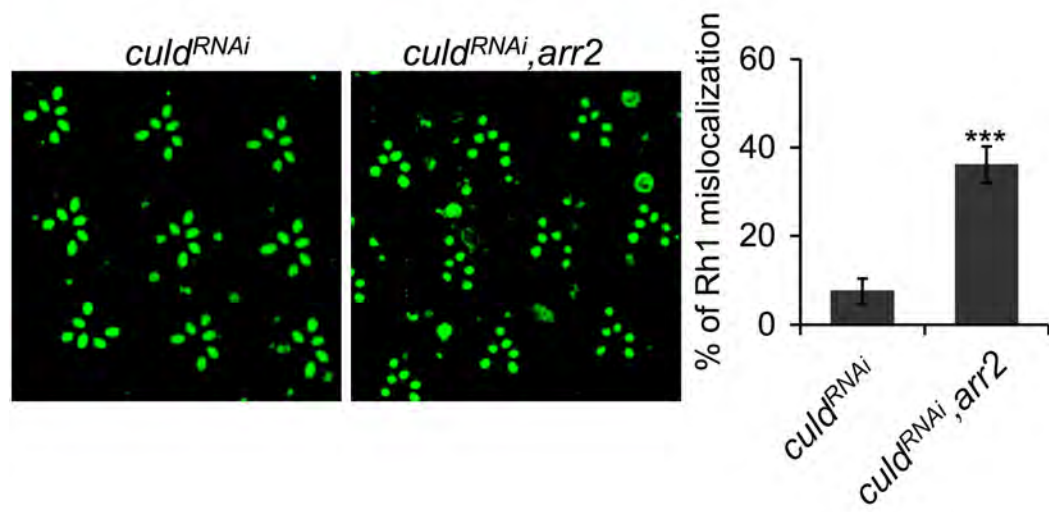


Figure 3.8

Figure 3.8. Knockdown of CULD expression through an *actin* promoter causes Rh1 mislocalization in both wild-type and an *arr2* mutant background.

A. Rh1 distributions in 7-day-old *Act5-gal4;UAS-culd^{RNAi}* (left) and *Act5-gal4;UAS-culd^{RNAi},arr2* (right) flies. The *culd^{RNAi}* was expressed through the *actin* promoter *Act5*. Both flies have two copies of *w⁺*, and thus have red eyes.

B. Quantification of the Rh1 mislocalization in the *culd* knockdown flies. The asterisk (*) indicates a significant difference from *culd^{RNAi}* (***: $P < 0.001$).

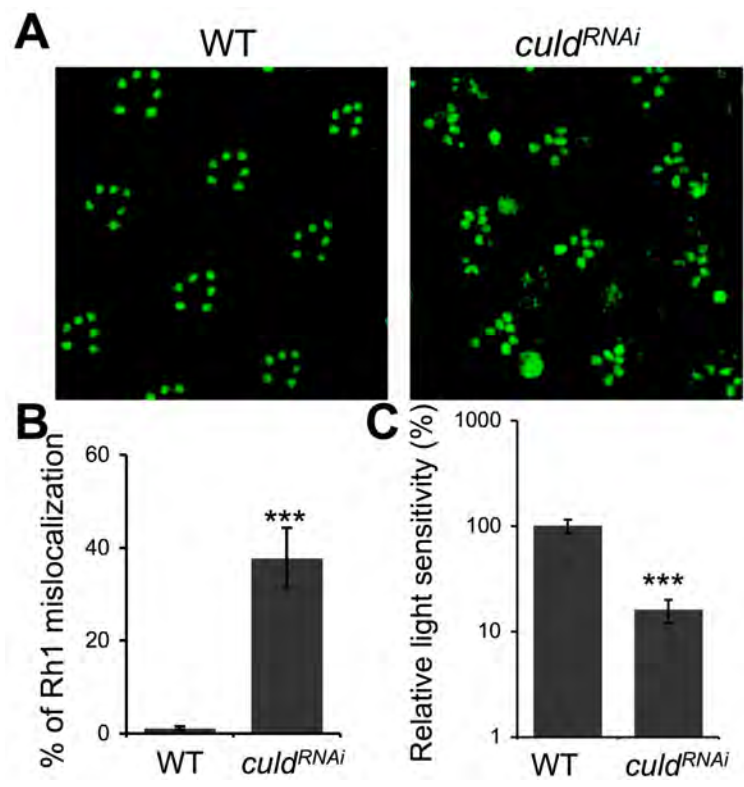


Figure 3.9

Figure 3.9. Knockdown of CULD expression through a *tubulin* promoter phenocopies the *culd* mutant.

A. Rh1 was mislocalized in a *cn,bw;tub-gal4/culd^{RNAi}* fly (right panel). This fly has white eyes due to *cn,bw*.

B. Quantification of Rh1 mislocalization in 4-day-old flies. The asterisk (*) indicates a significant difference from the wild type (***: $P < 0.001$).

C. Quantification of ERG sensitivities in 4-day-old flies. The asterisk (*) indicates a significant difference from the wild type (***: $P < 0.001$).

CHAPTER IV: GENERAL DISCUSSION

The work presented in this dissertation is focused on the molecular mechanisms underlying the maintenance of high photoreceptor sensitivity in *Drosophila*. Our studies have identified two molecules that are required to maintain the sensitivity through different pathways. First, TADR prevents an abnormal activity of G_q to maintain the rhabdomeral membrane structure. Second, CULD antagonizes Arr1-mediated Rh1 rhodopsin endocytosis to keep the rhabdomeral localization of Rh1, thereby maintaining the photoreceptor sensitivity.

4.1 New insights into the mechanisms underlying the maintenance of photoreceptor sensitivity

The high sensitivities of photoreceptors depend to a great extent on a high content of rhodopsin in light sensory organelles, which is achieved by the following two mechanisms.

First, tightly organized microvilli greatly increase the photoreceptive membrane area to accommodate a huge amount of rhodopsin molecules. Previous studies have shown that activities of Rh1 and TRP need to be controlled appropriately to prevent rhabdomeres from degeneration, which occurs, at least partially, due to over stimulation of phototransduction cascade (Iakhine et al., 2004; Dolph et al., 1993; Alloway et al., 2000; Yoon et al., 2000; Raghu et al., 2000b). In this thesis, we have shown that the signaling of G_q protein also needs to be regulated to prevent rhabdomeral degeneration and TADR promotes the translocalization of activated G_q from rhabdomeral membrane to cytosol. In the absence of TADR, the activated G_q is retained on the rhabdomeral membrane, thereby triggering the degeneration of rhabdomeres. Surprisingly, the downstream effectors of G_q in phototransduction pathway, including PLC and TRP, are not involved in the degeneration, suggesting that G_q activates a branched pathway to induce the rhabdomeral degeneration.

Second, to achieve the high rhodopsin content in rhabdomeres, the concentration of rhodopsin in rhabdomeral membrane has to be high. However, *Drosophila* Rh1 rhodopsin undergoes activity-dependent endocytosis through Arr1-mediated pathway in wild-type flies, at least at the pupal stage (Sato and Ready, 2005). Nonetheless, the level of endocytosed Rh1 is very low in adult flies, which does not significantly decrease the Rh1 concentration in rhabdomeral membrane. In this thesis, we have demonstrated that a new protein CULD that antagonizes Arr1-mediated, light-dependent endocytosis of Rh1 rhodopsin, at least partially through promoting recycling of endocytosed rhodopsin molecules. This provides *in vivo* evidence to show that endocytosed Rh1 undergoes recycling, which recovers the rhodopsin concentration in the rhabdomeral membrane.

4.2 Significance to the study of general GPCR signaling

GPCRs form the largest family of cell-surface receptors and mediate a wide variety of biological processes, including vision, taste and smell (Ferguson, 2001; Claing et al., 2002; Shenoy and Lefkowitz, 2003). Binding to the ligand triggers a conformational change in the receptor, which leads to the stimulation of heterotrimeric G proteins (Alberts et al., 2002). Based on the amino acid sequence relatedness of the α subunits, G proteins can be divided into three major families. It used to be thought that the G_q family protein only activates phospholipase C (PLC) (Alberts et al., 2002). However, recent *in vitro* studies suggested that G_q can activate alternative pathways via small GTPase, such as Rho and ARF (Giguère et al., 2006; Lutz et al., 2005; Rojas et al., 2007). Our study on the *tadr* mutant has provided *in vivo* evidence to show the existence of branched pathways. This study also points to the importance of the G_q localization in the determination of the downstream effectors.

To ensure that the extracellular stimuli are translated into intracellular signals with ap-

appropriate magnitude and specificity, GPCR signaling cascade is tightly regulated. One of the major mechanisms is to modulate GPCR endocytic trafficking, which controls the amount of cell surface receptors (Claing et al., 2002; Moore et al., 2007). While some endocytosed GPCRs are sorted to lysosome for degradation, many others are recycled back to plasma membrane for the cell resensitization (Claing et al., 2002). Nonetheless, the mechanism that underlies GPCR recycling remains largely unknown. Our study on the *culd* mutant has shown the GPCR recycling in an intact organism. Most importantly, we have identified CULD as a membrane protein that mediates the recycling of the GPCR/Rh1. The interaction between CULD and Arr1 is important for the CULD function *in vivo*. Further studies on the CULD-Arr1 and Arr1-Rh1 interactions may lead to the unveiling of a molecular mechanism of how CULD promotes Rh1 recycling.

4.3 Clinical relevance

1) Retinal disorders

The *Drosophila* eye is a valuable model system for the study of genetic, molecular and cell biological bases of retinal disorders. People have successfully modeled autosomal dominant retinitis pigmentosa (ADRP) with *Drosophila*. Proline substitution at position-23 by histidine (P23H) is the most frequent rhodopsin mutation causing ADRP (Galy et al., 2005). Although transgenic mice and rats expressing RhoP23H recapitulate dominant photoreceptor degeneration (Olsson et al., 1992), it is hard to clarify the pathogenic mechanism using these models. The powerful genetics makes *Drosophila* a better system to study the pathologies of human disorders at the molecular level. The Rh1P37H transgenic fly faithfully reproduces the pathological events occurring in ADRP patients that carry the RhoP23H mutation: age-/light-dependent dominant photoreceptor degeneration and vision

loss. More importantly, this *Drosophila* model has contributed to the clarification of the pathogenic mechanism of the disease: apoptotic death through the activation of two stress-specific mitogen-activated protein kinases (MAPKs), p38 and JNK (Galy et al., 2005).

Our study on the *tadr* mutant suggests that the G_q -mediated photoreceptor degeneration is through a branched pathway that is different from the phototransduction cascade. Similar alternative pathways of G protein may also exist in mammalian photoreceptors. Rac1, a potential effector of transducin, has been shown to mediate the photo-oxidative stress-induced photoreceptor degeneration in mammals (Haruta et al., 2009). Thus, further characterization of the G_q -mediated rhabdomeral degeneration in *Drosophila* photoreceptors may provide valuable clues for the therapy of retinal degeneration disorders in human.

2) G protein-mediated neuronal degeneration

An increasing number of studies show that G proteins are implicated in the pathophysiology of neuronal degenerative disorders. Alzheimer's disease (AD) is the most frequent neuronal degenerative disease of older Americans (Thathiah and Strooper, 2009). In some AD patients, beta-amyloid accumulation has an effect on the oligomerization of the angiotensin II type 2 receptor and sequestration of the $G_{\alpha_{q/11}}$ family of G proteins (Thathiah and Strooper, 2009; AbdAlla et al., 2009b). The sequestration of $G_{\alpha_{q/11}}$ causes neuronal degeneration, which accounts for the AD pathology (Thathiah and Strooper, 2009; AbdAlla et al., 2009b; AbdAlla et al., 2009a). Meanwhile, the $G_{\alpha_{q/11}}$ sequestration results in the dysfunctional coupling and signaling between M1 mAChR and $G_{\alpha_{q/11}}$, which prevents the AD pathology (Thathiah and Strooper, 2009; AbdAlla et al., 2009a).

Our study of the *tadr* mutant suggests that dysregulation of G_q protein localization activates branched pathways to induce photoreceptor degeneration. This further supports the theory that mislocalization of G_q proteins may trigger neuronal degeneration in different organisms. Thus, identification of alternative effectors of G_q protein in neurons may pro-

vide potential targets for therapeutic treatments of neurodegenerative disorders including AD.

3) GPCR endocytic trafficking and drug tolerance

Numerous GPCRs undergo endocytosis upon stimulation. And many of them are recycled back to the membrane after the stimulating ligand is removed (Claing et al., 2002). The failure of GPCRs in this cycle will change the receptor densities on the cell surface and affect the cell sensitivities to environmental stimuli. The failure of this process may also lead to other clinical problems, such as drug tolerances (Alvarez et al., 2001; Finn and Whistler, 2001). It has been reported that endocytosis of the mu opioid receptor (MOR) can reduce the development of morphine tolerance in rat (Finn and Whistler, 2001).

Our study on the *culd* mutant has identified a protein CULD that is required to promote Rh1 recycling, and the interaction with Arr1 is important for its function. Similar mechanisms may regulate the endocytic trafficking of non-visual GPCRs, such as MOR. Thus, CULD-related proteins in mammals are potential molecular targets for the clinical treatments to potentiate the receptor recycling and to alleviate drug tolerance.

4.4 Future studies

In this thesis we have identified new molecular mechanisms underlying the maintenance of visual sensitivity in *Drosophila*, either through preventing G_q -mediated rhabdomeral degeneration or through antagonizing arrestin-mediated rhodopsin endocytosis. However, the molecular components involved in these pathways are still unclear. To identify the molecular pathways, we will first examine some known-function molecules.

As the branched pathway of G_q induces *tadr*-mediated rhabdomeral degeneration, we will test alternative effectors of G_q to investigate whether they are required for the degen-

eration. ARF- and Rho-GEF proteins could be such candidates (Giguère et al., 2006; Lutz et al., 2005; Rojas et al., 2007).

To further understand the Rh1 recycling pathway, we will examine the function of known factors that are critical for GPCR recycling, such as Rab11 and Rab4 (Seachrist and Ferguson, 2003). Although Rab11 mediates post-golgi trafficking of Rh1 during development (Satoh et al., 2005), its role in the recycling of rhodopsin remains unknown. We may test this by examining Rh1 localization and visual sensitivity in Rab11 or Rab4 knock-down flies. It is also interesting to investigate whether Rab11 and/or Rab4 work together with CULD to promote Rh1 recycling by co-labeling Rab11 and/or Rab4 with CULD in Rh1 positive endocytic vesicles. Furthermore, we may test the role of Rab11 in Rh1 recycling by overexpressing the gene of *blue cheese* (*bchs*), whose product is expressed in the *Drosophila* eye (Finley et al., 2003) and antagonizes Rab11 function (Khodosh et al., 2006). In addition to Rab11 and Rab4, SNARE proteins mediate vesicle fusion and are known to be involved in vesicle recycling (Igarashi and Watanabe, 2007). We may test their function in the Rh1 recycling process.

To better understand the mechanisms required for the maintenance of visual sensitivity, it is important to identify the complete genetic network that is critical for the sensitivity. For this purpose, we can conduct an unbiased, genome-wide genetic screen. Conventional screens for visual genes assayed homozygous mutant flies, and thus missed the genes that are essential for the fly to survive to adulthood. In order to assay visual functions of these genes and provide a complete picture of the gene network that controls visual signaling, we will introduce a mosaic technique to an ethyl methanesulfonate (EMS)-based mutagenesis screen as described by Stowers and Schwarz (Stowers and Schwarz, 1999). In this case, we can detect recessive visual phenotypes in heterozygous mutant flies. We will conduct the screen in a genetic background that expresses a GFP-fused Rh1, so that we can assay

the rhabdomeral structure in live flies by examining the Rh1 expression pattern under fluorescence microscope. We may also directly assay the visual sensitivities of mutant flies with ERG recording. Figure 4.1 shows an example of genetic crossing scheme to screen for mutant genes on the arm 2L.

Based on this screen, we hope to get more mutants with abnormal rhabdomeral structure. Once the mutant gene is mapped and identified, we will characterize how loss of the gene leads to the degeneration. Specifically, we will investigate whether a signaling component of visual transduction cascade is involved in the degeneration, as we did for the *tadr* mutant. Finally, we will attempt to identify factors that mediate the degeneration downstream of that visual signaling molecule.

From this screen, we also hope to get some mutants with *culd* phenotypes, including reduced visual sensitivity and abnormal rhodopsin localization. Once the mutant gene is mapped and identified, we will characterize whether the protein, encoded by the gene, is involved in Rh1 endocytic trafficking. Importantly, we will investigate how this protein antagonizes Rh1 endocytosis to maintain photoreceptor sensitivity. Specifically, we will examine whether this protein works together with CULD to promote Rh1 recycling. These studies will advance our understanding of Rh1/GPCR endocytic trafficking and its physiological functions.

Moreover, we can screen for the components involved in *tadr*-mediated retinal degeneration and *culd*-mediated Rh1 mislocalization through modifier screens (Johnston, 2002). We will use a similar protocol to that in Fig 4-1. The only difference is that all flies used in the screen have a *tadr* or *culd* mutant background. We will screen for fly lines with enhanced or suppressed original phenotypes to fully understand the molecular pathways.

In summary, the studies detailed in this dissertation have identified two essential molecular mechanisms underlying the maintenance of high rhodopsin content in photosensory

organelles, which is required for the achievement of continued high visual sensitivity. The identification of G_q -mediated rhabdomeral degeneration through a branched pathway has provided new molecular basis for rhabdomeral protection. The discovery of a CULD-mediated post-endocytic recycling pathway of Rh1 rhodopsin has solved a long-standing puzzle about how photoreceptors antagonize arrestin-dependent Rh1 rhodopsin endocytosis to maintain high visual sensitivities. This work advances our understanding of visual biology and the regulation of GPCR signaling in general, and may provide valuable clues to pathologic studies of human retinal degeneration disorders, such as RP, which may help to reveal therapeutic approaches.

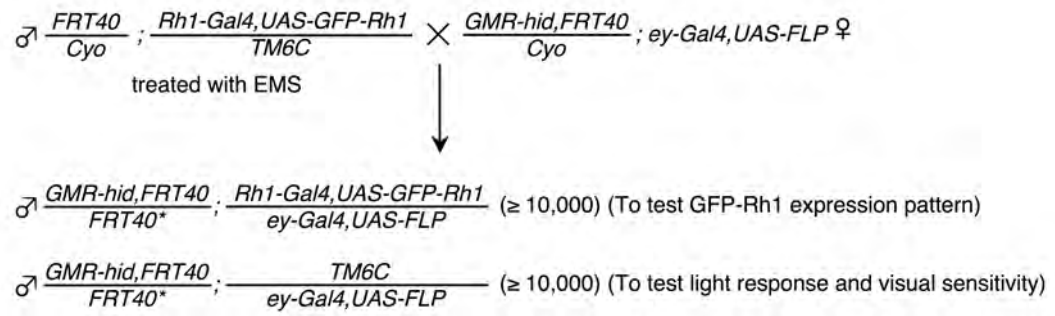


Figure 4.1

Figure 4.1: Genetic crossing schemes to look for causal genes of retinal disorders by EMS screen.

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